V. 12.0

Signal-Seeker™ SUMOylation 2/3 Detection Kit

30 Assays

Cat. # BK162

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I: Introduction: Overview and Applications

Overview

Signal-Seeker™ kits offer end users a powerful set of tools for characterizing key protein modifications (also termed post-translational modifications or PTMs) involved in the regulation of any protein of interest (POI). Used individually, Signal-Seeker™ kits can give insight into a protein's function at a level of detail unlikely to be achieved with standard characterization or proteomic approaches. As Signal-Seeker™ kits have been designed to work together, end-users can combine data from different Signal-Seeker™ kits to generate an unbiased snapshot of protein PTM cross talk; ultimately, contributing to the exciting and rapidly growing field of protein regulation (1-4). For the full range of Signal-Seeker™ kits please visit www.cytoskeleton.com.

Applications

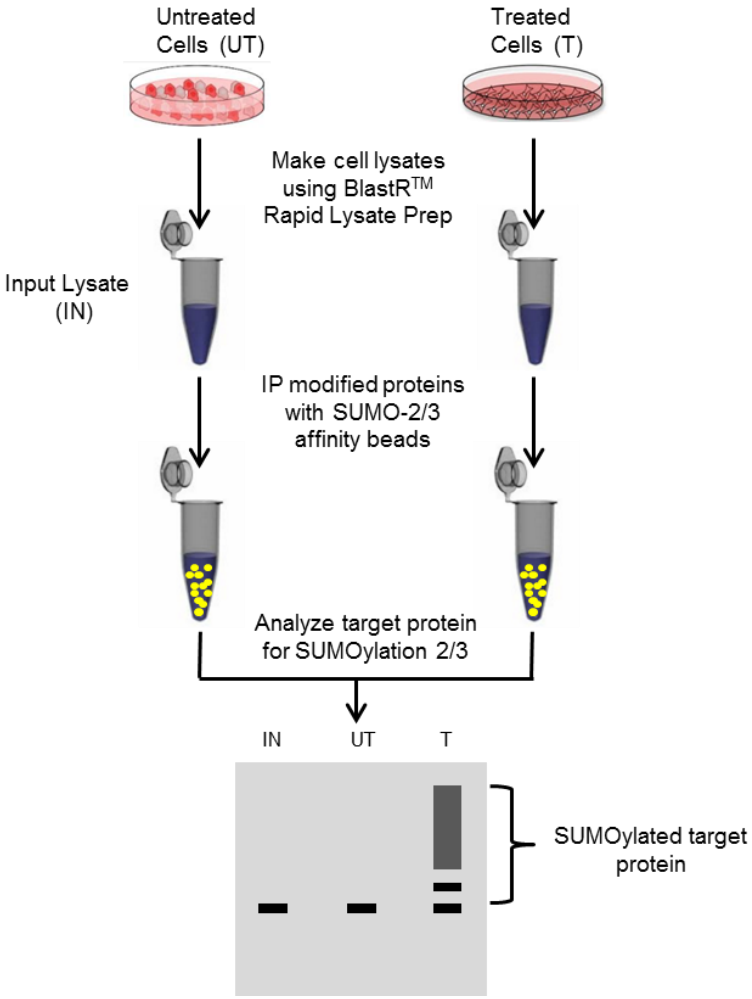
- Discover and publish novel regulatory mechanisms.
- Detect highly transient regulation of protein modifications.
- Confirm data generated from transfection or proteomic approaches.
- Use a selection of Signal-Seeker™ kits to build a PTM profile for your POI.
- Investigate the role of known protein modifications in your system.
- Endogenous protein PTM detection is most likely to give biologically relevant data.
- Discover novel biomarkers.

I: Introduction: Assay Principle

Assay Principle

Signal-Seeker™ kits use affinity beads to pull-out and enrich modified proteins from any given cell or tissue lysate. The enriched protein population is then analyzed by standard western blot procedures and the modified protein of interest is detected by the end-user using their own primary antibody (Figure 1). Signal-Seeker™ kits are available for several key PTMs, including phosphorylation (phosphotyrosine), ubiquitination, SUMOylation and acetylation (see www.cytoskeleton.com for the full range of kits). These kits have been designed to work together to allow a PTM profile to be generated from a single lysate.

Figure 1: Schematic showing Signal-Seeker™ Assay Flow



II: Kit Contents: Reconstitution and Storage

This kit contains enough reagents for 30 SUMO 2/3 assays and 10 control assays. Store entire kit at 4°C until initial use. Prior to beginning the assay, you will need to reconstitute several lyophilized components as shown in Table 1. When properly stored and reconstituted, components are guaranteed stable for 6 months.

Table 1: Component Storage and Reconstitution

Kit Component	Cat. # /Part # (Quantity)	Reconstitution	Storage after reconstitution
BlastR™ Lysis Buffer	Part# BLST01 2 bottles	Resuspend in 15 mls sterile water to give a 1X stock solution	4°C
BlastR™ Dilution Buffer	Part# BDB01 1 bottle	Resuspend in 130 mls sterile water to give a 1X stock solution.	4°C
BlastR-2™ Wash Buffer	Part# BWB02 1 bottle	Resuspend in 130 mls sterile water to give a 1X stock solution.	4°C
De-ubiquitination/ SUMOylation Inhibitor (N-ethylmaleimide & TPEN)	Part# NEM09BB 2 tubes	1) Resuspend in 500 µl of DMSO (provided in kit) for a 100X stock solution. 2) Aliquot 5 x 100 µl volumes. This reduces freeze thaw cycles.	-20°C
Protease Inhibitor Cocktail	Cat# PIC02 1 tube	Resuspend in 1 ml of DMSO (provided in kit) for a 100X stock solution	-20°C
SUMO-2/3 Affinity Beads	Cat# ASM24-Beads 3 tube	Resuspend in 440 µl of 50% glycerol. Each IP assay uses 40 µl of bead slurry.	-20°C
IP Control Beads (to assess non-specific binding)	Cat# CIG01-Beads 2 tube	Resuspend each tube in 330 µl of 50% glycerol. Each IP assay uses 40 µl of bead slurry. NOTE: there will be excess control beads	-20°C
Anti-SUMO-2/3-HRP antibody	Cat# ASM23-HRP-S (1 tube)	Resuspend in 25 µl of water	4°C
Precision Red™ Advanced Protein Assay Reagent	Part #GL50 1 bottle (100 ml)	Not required	Room temp.
Bead Elution Buffer	Part# BEB01 1 tube (1.3 ml)	Not required	Room temp.
DMSO	Part# DMSO 2 tubes (1.5 ml)	Not required	Room temp.
Chemiluminescent detection reagent A	Part # CLRA 1 bottle (10 ml)	Not required	Room temp.
Chemiluminescent detection reagent B	Part# CLRB 1 bottle (10 ml)	Not required	Room temp.
Spin columns Collection tubes	Part# SPN22 40 of each	Not required	Room temp.
BlastR™ filters	Cat# BLR02 30 filters	Not required	Room temp.

Items with catalog numbers (Cat. #) are available separately.

Standard reagents and equipment that you will require but are not supplied:

- Tissue culture cells or tissue of interest
- PBS pH 7.4 buffer (10 mM phosphate buffer pH 7.4, 140 mM NaCl, 3 mM KCl)
- Cell scrapers
- Liquid nitrogen for snap freezing cell lysates
- 2 mercaptoethanol
- SDS-PAGE system and buffers
- PVDF, Western transfer system, and buffers
- Blocking reagent
- Primary antibody to target protein
- HRP-labeled secondary antibody
- Chemiluminescence documentation instrument

STEP 1A: Sample Preparation: Cell Culture

It is recommended to aim for 1.0 mg of total protein lysate per IP assay as an optimal starting point. We recommend utilizating150 cm^2 plates.

Protein yield varies widely for any given cell line, and it is strongly recommended to perform a "test plate" protein quantitation, particularly if you are unsure of the expected protein yield from your experimental conditions (see appendix V).

Processing Tissue Culture Cell Lysate:

1. Grow and treat tissue culture cells as required.
2. Prepare BlastR™ lysis and dilution buffers with inhibitors (see appendix VI).
3. Remove culture media and wash the cells twice with 10 ml of 1x PBS. Note: remove as much PBS as possible prior to adding BlastR™ lysis buffer in order to maximize cell lysis.
4. Add appropriate volume of BlastR™ lysis buffer (based on expected protein yield; see table 2) and lyse cells using a cell scraper. The lysate will become viscous due to nuclear lysis.

Table 2: BlastR™ Lysis/Dilution Buffer Chart

Plate Protein content	Recommended BlastR™ Lysis Buffer volume	Recommended BlastR™ Dilution Buffer volume
< 1 mg	Combine protein from multiple plates: See appendix VIII, step 9	To make 1.5 ml final volume
1-2 mg	300 µl	To make 1.5 ml final volume
2-4 mg	600 µl	To make 3 ml final volume
4-6 mg	900 µl	To make 4.5 ml final volume

5. Use a snipped 1 ml pipette to transfer the crude lysate into a BlastR™ filter that has been placed in a 15 ml collection tube (see Appendix VII).
6. Use a supplied filter plunger to completely compress the BlastR™ filter and collect the lysate flow through, including any bubbles , in a 15 ml tube (see Appendix VII).

NOTE: At this point the lysate volume should not exceed 2x the original lysis buffer volume. This will occur due to incomplete removal of PBS during the wash step.

7. Optional: Centrifuge the lysate at approximately 10,000 g for 1 minute at 4°C. Transfer to a new tube.
8. Dilute the lysate with BlastR™ Dilution Buffer to give the final volume given in Table 2. This step is important as the final buffer composition will affect the IP reaction stringency.
9. Quantitate protein concentration (see appendix VIII).
10. Based on protein concentration, dilute sample with a buffer mix (1 part BlastR™ lysis: 4 parts BlastR™ dilution) to a desired final concentration (usually 1 mg/ml).
11. Snap freeze aliquots of any samples that won't immediately be used. Proceed to STEP 2.

STEP 1B: Sample Preparation: Tissue

It is recommended to aim for 1.0 mg of total protein lysate per IP assay as an optimal starting point. Table 3 provides examples of protein yield from 100 mg of various tissue types.

Table 3: Example: Total Protein Obtained From 100 mg of Tissue

Tissue Type	Tissue amount (mg)	Total Protein (mg)
Liver	100mg	10-15
Heart	100mg	10-20
Brain	100mg	8-12

Processing Tissue Lysate:

1. Obtain fresh or frozen tissue.
2. Prepare BlastR lysis and dilution buffers with inhibitors (see appendix VI).
3. Add 1 ml of BlastR™ lysis buffer per 100 mg of tissue in an appropriate-sized, glass dounce tissue grinder/homogenizer, and apply 10-12 strokes. The lysate will become viscous due to nuclear lysis.

   Note: The amount of BlastR™ lysis buffer will change depending on the amount of tissue used. For example, if you use 50 mg of tissue then lyse with 500 µl of BlastR™ lysis buffer.

5. Use a snipped 1 ml pipette to transfer the crude lysate into a BlastR™ filter that has been placed in a 15 ml collection tube (see Appendix VII).
6. Use a supplied filter plunger to completely compress the BlastR™ filter and collect the lysate flow through, including any bubbles , in a 15 ml tube (see Appendix VII).
7. Add 200 µl of filtered lysate to a 1.5 ml tube. Snap freeze the remaining lysate.

   Note: Only a fraction of lysate is processed further as 100mg of tissue will produce excess lysate. However, this is tissue type dependent.

8. Dilute the 200 µl of filtered lysate with 200 µl of BlastR™ Dilution Buffer and mix.
9. Centrifuge the lysate at approximately 10,000 g for 10 minute at 4°C in a table-top microcentrifuge. Transfer supernatant to a new 15 ml tube.
10. Add 600 µl of BlastR™ Dilution Buffer to dilute the clarified lysate to a 1 ml final volume. This step is important as the final buffer composition will affect the IP reaction stringency.
11. Quantitate protein concentration (see appendix VIII).
12. Based on protein concentration, dilute sample with a buffer mix (1 part BlastR™ lysis: 4 parts BlastR™ dilution) to a desired final concentration (usually 1 mg/ml).
13. Snap freeze aliquots of any samples that won't immediately be used. Proceed to STEP 2.

STEP 2: Immunoprecipitation (IP) Assay

1. Invert tube containing SUMO 2/3 Affinity Bead suspension several times to make sure that the beads are completely resuspended in the tube.
2. For each IP assay, aliquot 40 µl of bead suspension into a tube on ice. (IP tube).
3. Invert tube containing IP Control Bead suspension several times to make sure that the beads are completely resuspended in the tube.
4. Aliquot 40 µl of bead suspension per control reaction to determine non-specific binding. (Control IP tube).
5. Wash beads 2 times with 250µl 1X PBS-T to remove storage buffer.
6. Save a small amount of lysate (20 µl) to run as a western input lysate control. Add 5 µl of 5x sample buffer and boil for 5min. Save for western analysis.
7. Add lysate to each IP tube and control IP tube. We recommend 1.0 mg of lysate per assay as a starting point. NOTE: the amount of lysate required will vary depending upon the abundance of modified target protein.
8. Incubate the tubes on a rotating platform at 4°C for 2h.
9. Collect beads by centrifugation at 3-5,000 x g for 1 minute at 4°C.
10. Aspirate off as much supernatant as possible without disturbing the beads.
11. Wash beads in 1 ml Wash Buffer (inhibitors are not necessary at this stage) for 5 minutes on a 4°C rotating platform.
12. Collect beads by centrifugation at 3-5,000 x g for 1 minute at 4°C.
13. Aspirate off as much supernatant as possible without disturbing the beads.
14. Repeat the wash step (10-12) two more times.
15. After the final wash, completely remove buffer supernatant. Minimal disruption of the bead pellet (5% loss) is acceptable. Recommended Technical Tip: remove residual supernatant using a fine bore protein loading tip.
16. Add 30 µl of Bead Elution Buffer and resuspend the beads by gently tapping/flicking the side of the tube. DO NOT use a pipette at this stage.
17. Incubate at room temperature for **exactly** 5 minutes.
18. Gently transfer each bead suspension to one of the spin columns provided in the kit. It is recommended to snip the end off the transfer pipette tip for gentler transfer.
19. Place the spin column in a fresh collection tube and centrifuge at 9-10,000 x g for 1 minute at room temperature to collect the IP sample.
20. Add 2 µl of 2-mercaptoethanol to each sample and mix well.

    NOTE: It is convenient to snap the lid off the spin column and use this to cap the collection tube for further processing.
21. Place samples in a boiling water bath for 5 minutes. Collect sample by centrifugation at 10,000 x g for 1 minute at RT.

22. If necessary, freeze samples and stop here, or proceed to running SDS-PAGE and western blot analysis, see STEP 3A & 3B.

Performing the IP assay with appropriate controls is critical to effectively analyze the results. In addition to the controls highlighted in this IP assay, please see Appendix IX for detailed information about recommended control reactions and experiments.

STEP 3: Western Blot Protocol

STEP 3A: Western Blot for Identification of Protein of Interest

Primary Antibody Incubation

A primary antibody provided by the end user will be used for detection of the SUMO 2/3 conjugated proteins of interest. The SDS-PAGE and western blot should be performed according to your laboratory protocol.

Secondary Antibody Recommendations

Signal-Seeker™ beads covalently link bead bound protein G to PTM affinity antibodies in order to prevent /minimize light and heavy chain leaching from beads during PTM enrichment. In some cases trace antibody leaching will result in the detection of light chain (approx. 30 kD), heavy chain (approx. 55 kD) and a high molecular weight complex of heavy/light/protein G complex (approx. 200 kD) by mouse secondary antibodies. Hence, for detecting mouse monoclonal primary antibodies (MAbs), we would recommend using a 1:1,000 dilution of an HRP-conjugated secondary antibody that preferentially recognizes native mouse primary antibodies and hence will not detect any contaminating denatured antibody present in the western blot. Anti-mouse-HRP TrueBlot ULTRA antibody from Rockland (Cat# 18-8817-30) is highly recommended. See Appendix X for example data. **NOTE:** we do not recommend HRP-protein G-based reagents such as CleanBlot as these lack the sensitivity required to detect most endogenous protein specific PTMs (see Appendix X).

Western Detection Reagent

While colorimetric and fluorescent detection methods may provide sensitive, linear western signals for the detection of your target protein, we highly recommend the use of the ultrasensitive chemilluminescence detection reagent that is supplied in this kit as it is generally 10 fold more sensitive than fluorescence detection and 20 fold more sensitive than colorimetric.

The chemiluminescent reagent should be used in conjunction with an HRP-labeled secondary antibody capable of detecting your primary antibody (see above for secondary detection reagent recommendations).

The chemiluminescent detection reagents supplied in this kit is sufficient for 10 mini-gel sized Westerns. A volume of 2 ml of chemiluminescent reagent per minigel sized transfer membrane (approx. 8 x 7 cm) should be used. The following method is recommended;

a) After incubation with appropriate secondary antibody (30 minutes room temperature is recommended), wash the blot 6 x 10 minutes in TBST (50 ml per wash per 8 x 7 cm membrane)

b) Immediately before use, mix 1 ml of chemiluminescent reagent A with 1 ml of chemiluminescent reagent B (sufficient for one 8 x 7 cm membrane).

c) Add chemiluminescent reagent to membrane and incubate with gentle rocking at room temperature for 5 minutes prior to visualization of protein signal using x-ray film or CCD camera imaging.

NOTE: shorter incubation time in the chemiluminescent reagent may be necessary for highly abundant proteins.

STEP 3B: Determination of total SUMOylated 2/3 species in the IP

It is good practice to check the IPs for total SUMOylated 2/3 species. This serves as a control assay to make sure that the IP reactions are efficiently enriching SUMOylated 2/3 proteins. Two methods for determining IP efficiency are given below.

Method 1: Re-probe Blot with anti-SUMO2/3-HRP

1. After detection of the protein of interest, the blot can be re-probed with the anti-SUMO2/3-HRP labeled antibody supplied in this kit. This allows a positive confirmation that SUMOylated 2/3 proteins have been selectively enriched using the SUMO-2/3 affinity beads.

   NOTE: Stripping the blot prior to probing is not necessary as the signal from total SUMO-2/3 conjugates will be significantly stronger than that from your protein of interest.
2. After a brief 10 minute wash in TBST at room temperature with shaking, incubate the membrane with a 1:4000 dilution of anti-SUMO2/3-HRP antibody diluted in TBST (2.5% non-fat milk) for 1 h at room temperature or overnight at 4°C with constant agitation.
3. Wash the membrane 6 times in TBST for 10 min each.
4. Immediately before use, mix 1 ml of chemiluminescent reagent A with 1 ml of chemiluminescent reagent B (sufficient for one 8 x 7 cm membrane).
5. Add chemiluminescent reagent to membrane and incubate with gentle rocking at room temperature for 1-2 minutes prior to visualization of total SUMOylated 2/3 species signal using x-ray film or CCD camera imaging.

Method 2: Run a Separate Blot with a small amount of IP reaction

The signal from the total SUMOylated 2/3 species IP will be very strong. It is therefore possible to keep a small volume (2-3 µl per IP) to run a separate western for the purpose of determining IP efficiency.

IV: Troubleshooting

Observation	Possible cause	Remedy
No target protein SUMOylation 2/3 detected	There are several possible reasons for this result; 1) The protein of interest is not SUMOylated under the conditions examined. As the Signal-Seeker™ kits are essentially discovery tools there is no guarantee that a particular modification will occur under a given condition. 2) Protein SUMOylation can be very rapid and transient and can therefore be missed. 3) The amount of modified protein is typically only a small percent of the total protein (1-2%).	1) Make sure that the affinity beads enriched for total SUMOylated species by using the anti-SUMO2/3-HRP antibody to analyze the IP reactions and see below. 2) A time course is often appropriate particularly if signal transduction pathways are being analysed. 3) It is important to make sure the primary antibody is able to detect low ng of the target protein. To determine if the antibody sensitivity could be an issue it is a good idea to run 2% of lysate input on the Western blot. If the antibody detects the unmodified protein from the input lane then sensitivity is unlikely to be an issue. Also make sure that the chemilluminescent detection reagent from the Signal-Seeker™ kit is being used.
Unmodified protein band detected in IP	SUMO-2/3 Affinity Beads may detect a band that corresponds to the unmodified target protein. This may be non-specific or caused by de-SUMOylation during enrichment.	1) Make sure the de-SUMOylation inhibitor (NEM) provided in the Signal-Seeker™kit is used during lysis in both the BlastR™ Lysis Buffer and the BlastR™ Dilution Buffer. It is also important to aliquot the NEM stock to minimize freeze/thaw of the inhibitor. 2) In rare cases the POI may dimerize as a modified/unmodified complex, resulting in a co-IP of unmodified protein. The BlastR™ buffer system has been designed to cause aggressive cell lysis and is designed to break up even strong (low ng Kd) protein:protein interactions. It is important, during cell lysis that the BlastR™ buffer is not diluted out by residual wash buffer. For example, if 300 µl of BlastR™ Lysis buffer is used to lyse cells the final volume of undiluted lysate should not exceed 600 µl. 3) As SUMO-2/3 modified protein will run at a higher molecular weight than unmodified protein (SUMO is 12 kD), the presence of a small amount of unmodified signal is generally not an issue. It is always important to run an input sample of the cell lysate for the detection of unmodified protein. 4) Always run a SUMO-2/3 IP Control Bead reaction to determine degree of non-specific binding.

Observation	Possible cause	Remedy
Faint bands visible at 200, 55, and 30 kDa acetyl-lysine Bead samples	Trace amount of antibody or antibody-protein G complex can sometimes leach off from the affinity beads and can occasionally be detected as non-specific bands by some anti-mouse-HRP secondary antibodies.	1) Run SUMO 2/3 Affinity Bead only sample that does not contain cell lysate. If the bands are coming from the beads then they will be visible in this sample. 2) When detecting mouse monoclonal primary antibodies (MAbs), use a 1:1,000 dilution of an HRP-conjugated secondary antibody that preferentially recognizes native mouse primary antibodies and will not detect contaminating denatured antibody. Anti-mouse-HRP TrueBlot ULTRA antibody from Rockland (Cat# 18-8817-30) is highly recommended. (see Appendix X). 3) Alternatively, try using a rabbit primary antibody as these will not react with the leached mouse antibody.

V: References

1. Wang, Y, et al. 2015. Sequential posttranslational modifications regulate PKC degradation. *Mol. Biol. Cell*. **27**: 410-420.

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4. Satija Y.K. & Das S. 2016. Tyr99 phosphorylation determines the regulatory milieu of tumor suppressor p73. *Oncogene* **35**: 513-5

5. Barysch S. et al. 2014. Identification and analysis of endogenous SUMO1 and SUMO2/3 targets in mammalian cells and tissues using monoclonal antibodies. *Nat Protoc*. **9(4):**896-909

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VI: Appendix I: Extended Overview

Overview

Signal-Seeker™ kits offer end users a powerful set of tools for characterizing key protein modifications (also termed post translational modifications or PTMs) involved in the regulation of any protein of interest. Used individually Signal-Seeker™ kits can give insight into a protein's function at a level of detail unlikely to be achieved with standard characterization or proteomic approaches. As Signal-Seeker™ kits have been designed to work together, end-users can combine data from different Signal-Seekers™ to generate an unbiased snapshot of protein PTM cross talk and contribute to an exciting and rapidly growing area in protein regulation (1-4). Table 4 gives a very brief selection of some recent publications in this area. It is now clear that all proteins are regulated by one or more post-translational modification, Signal-Seekers™ allow you to quickly and simply assess the relevance of key PTMs such as phosphorylation, ubiquitination, acetylation and SUMOylation to your protein or pathway of interest (see www.cytoskeleton.com for the full range of kits).

One lysate, one day, huge insight

Table 4: Examples of PTM Cross-Talk in response to a given stimulus or physiological state

Protein	Co-dependent Modifications	Function	Ref.
Protein Kinase C (PKCα)	Phosphorylation SUMOylation Ubiquitination	**Observation:** PKCα is degraded over a 4h period following PKCa activation by the phorbol ester PMA. **Mechanism:** Mediated via a cascade of PTMs. A timecourse for PMA treatment showed the following series of events; a) Reduction in phosphorylation. b) Increased de-SUMOylation. c) Followed by an increased ubiquitination and ultimately PKCα degradation.	1
Tau	Hyperphosphor-yation SUMOylation Ubiquitination	**Observation:** SUMOylated Alzheimer's disease (AD) tau was observed in late stage Alzheimer's, this correlated with reduced clearance of AD-tau via the ubiquitin proteasome system. **Mechanism:** Data supports a cascade of PTM events; a) SUMOylation on tau induces hyperphosphorylation. b) Tau hyperphosphorylation enhances SUMOylation. c) SUMOylation inhibits tau ubiquitination and degradation of AD-tau.	3
p73	Phosphorylation Ubiquitination	**Observation:** Genotoxic stress induces an increase in p73 levels which allow it to mediate the stress response through apoptosis. **Mechanism:** Mediated via interplay of ubiquitination and phosphoprylation; a) Normal conditions promote p73 ubiquitination and degradation. b) Genotoxic stress promotes phosphorylation which inhibits ubiquitination and stabilizes p73.	4

VI: Appendix II: Assay Features

Assay Features

The study of endogenous PTMs poses several technical challenges (see Table 5). Signal-Seeker™ kits have been developed to give end-users the ability to quickly and easily look for PTM regulation in their protein/system of interest. They can also be used to confirm results obtained through proteomic or transfection studies. Table 5 describes several of the Signal-Seeker features that were developed to create a robust assay that can be used by PTM specialists and non-specialists alike.

Table 5: Assay Features

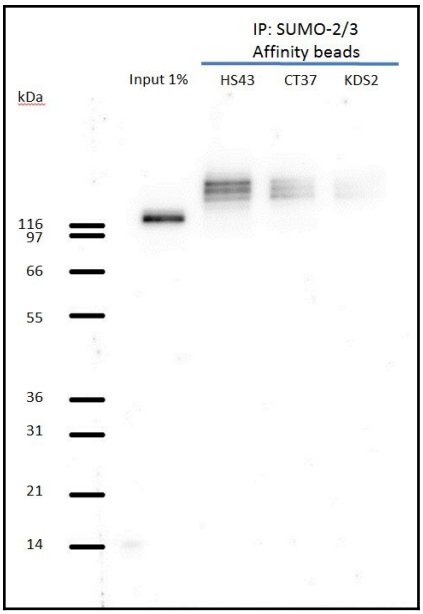
Technical Challenge	Signal-Seeker™ Solution
The percentage modified vs unmodified protein under any physiological condition is typically very low (modified being only 1-2% of the unmodified protein amount). This is reflective of the fact that modifications often occur at the site of action in the cell to localize the cellular response. In this way PTMs are similar to activation of small G-proteins such as Ras and Rho in which the active (GTP-bound) form of the protein only represents 1-2% of total Ras or Rho. The low level of modified protein is therefore disproportionate to its role in a physiological response and this makes detection of modified species difficult.	Optimized sensitivity is a key feature of Signal-Seeker™ kits. a) Validation studies have demonstrated that Signal-Seekers™ can detect low level endogenous protein modifications (see Example Data section). b) High affinity IP beads and an optimized proprietary buffer system (BlastR™) have been developed by scientists at Cytoskeleton Inc. to enhance assay sensitivity (see below). c) High sensitivity chemiluminescent detection reagents, capable of detecting fg levels of protein, have been included in this kit.
Because PTMs elicit strong cellular responses from the target protein the PTM events are tightly regulated and often very transient. This is particularly true in signal transduction pathways where a given PTM cycle (addition and removal) may be over in minutes. The transient nature of many PTMs make them difficult to capture.	a) TheSignal-Seeker™ kits have been optimized to detect very low levels of modified proteins. b) Validation studies have shown that the kit can detect low level transient PTM signals c) Clear assay instructions stress the importance of experimental design to capture key timepoints.
Affinity reagents may not capture all modified species in any given lysate. Proteomic studies have shown that different commercially available affinity matrices show quite different PTM capture profiles raising questions regarding their specificity and their comprehensiveness.	The affinity matrices used in Signal-Seeker™ kits have been developed in house by scientists at Cytoskeleton Inc. Our validation studies have consistently shown that Cytoskeleton's affinity reagents outperform other "best-in-class' commercial beads in IP applications. For detailed information see specific bead descriptions in this manual and visit www.cytoskeleton.com.
Buffer conditions are not compatible between different modifications.	Signal-SeekerTM kits contain a proprietary BlastR™ Rapid Lysate Prep system consisting of a Lysis buffer, Dilution buffer, and patented Filter. The buffer system was designed to work well with multiple PTM types, including SUMOylation, phosphorylation, ubiquitination and acetylation.
PTM enrichment experiments are complex and exacting. They require high quality affinity matices, optimized buffers and inhibitors and sensitive detection reagents.	Signal-Seeker™ kits have been optimized to give end-users the best chance of detecting target protein PTMs. The kits are accompanied by detailed and user-friendly instruction manuals and all of our products are supported by a knowledgeable technical support staff.

VI: Appendix III: SUMO 2/3 Affinity Beads

Signal-Seeker™ SUMO-2/3 Affinity Beads

Monoclonal mouse antibody (clone: 11G2) was developed and manufactured at Cytoskeleton Inc. (cat.# ASM24). The antibody was raised against full-length recombinant SUMO-2 protein (Uniprot: P61956), combined with a proprietary mix of peptides that include CQIRFRFDGQPINE. The linear epitope has not been identified and the antibody seems to recognize a conformational epitope. SUMO-2/3 affinity beads (cat.# ASM24-Beads) consist of the anti-SUMO-2/3 antibody (clone: 11G2) that has been chemically conjugated to Protein G beads. The affinity bead has been shown to immunoprecipitate a wide range of SUMO-2/3 targeted proteins in A431 cell lysate (Fig. 3). The affinity bead reagent has been optimized to give no detectable leaching of either heavy or light chains in an IP assay, making the resulting data extremely specific, sensitive and clean (Fig. 2 & 3). The detection of endogenous mono– and poly-SUMOylated TFII-I proteins from HeLa cells is visualized with characteristic multiple bands in Western Blot (Fig. 2). This representitive data agrees with published data and demonstrates the utility of SUMO-2/3 affinity beads and Signal-Seeker™ kits in studying the rapidly growing area of SUMO modification of proteins (5).

Figure 2: Detection of SUMO-2/3 conjugated TFII-I in HeLa cell lysate by using SUMO-2/3 Affinity Beads.

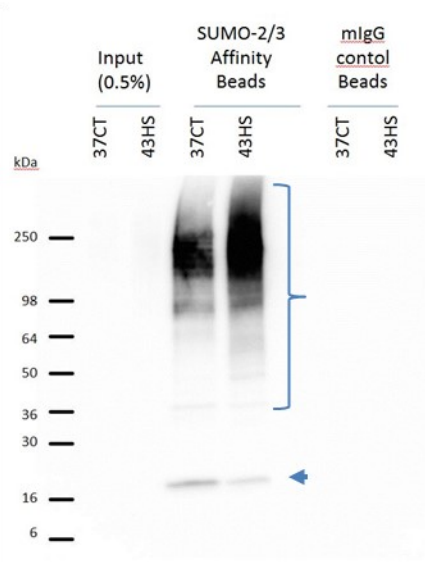


Denatured HeLa cell lysates were prepared from HS43, CT37 and KD S2 (HS43: Heat Shock treated (43°C for 10min), CT37: untreated and KD S2: shRNA SUMO-2 knock down). 1mg of lysate was used for the immunoprecipitation of SUMO-2/3 conjugates. IP experiment was performed according to the presented protocol. Western blots of immunoprecipitated proteins were developed using anti-TFII-I antibody (Abcam). The level of SUMO-2/3 conjugated TFII-I proteins in heat shock treated cells is higher than control and shRNA SUMO-2 knock-down reduced the level of the conjugates. Chemical conjugation of 11G2 to agarose beads prevents heavy and light chain leaching. Unconjugated TFII-I is visible near 120kDa (Input lane). High molecular-weight bands indicate that TFII-I is conjugated by multiple SUMO-2/3 proteins. TFII-I has previously been reported to be a target for SUMOylation(6).

VI: Appendix IV: Example Data

The data shown below was generated using the Signal-Seeker™ SUMO-2/3 Enrichment kit. The target proteins detected are from endogenous total proteins and endogenous p53 respectively.

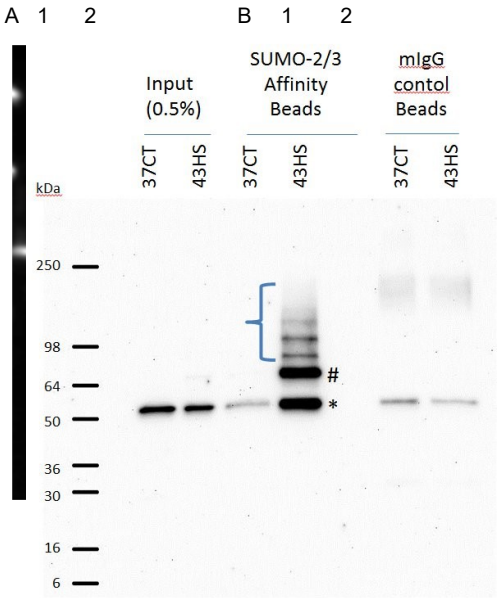
Figure 3A: Enrichment of SUMO-2/3 conjugates



A431 cell lysates were prepared as described in this protocol from HS43 and CT37 (HS43: Heat Shock treated (43°C for 10min), CT37: untreated). SUMO-2/3 conjugates were enriched from 1mg of lysate and immuno-blotted by anti-SUMO-2/3 antibody (Cytoskeleton cat# ASM23) (Fig. 3A) and anti-p53 antibody (Sigma, DO-1) (Fig. 3B) along with control beads.

The level of total SUMO-2/3 conjugates in heat shock treated cells (Fig 3A: SUMO 2/3 Affinity Beads Lane 43HS) is stronger than control cells (Fig. 3A: SUMO 2/3 Affinity Beads Lane 37CT). Total SUMOylated protein signal is delineated by the blue bracket, the blue arrowhead indicates the position of free SUMO 2/3.

Figure 3B: Enrichment of SUMO-2/3 p53 protein



The well-studied SUMO-2/3 target protein, tumor suppressor p53 was immuno-blotted (Fig. 3B). Pound (#) and bracket({) indicate mono- and poly- SUMOylated p53 proteins, respectively. SUMO-2/3 unmodified p53,denoted by star(*) and may be due to non-specific bead binding (see control bead lanes) or de-SUMOylation events during immunoprecipitation.

HeLa cells were grown to 70% confluency and harvested by lysis in BlastR™ buffer. Lysates (500 µg per assay) were treated as outlined in the Signal-Seeker™ Ubiquitination Enrichment manual. The western

Test Plate to Determine Protein Concentration

It is recommended to aim for 1.0 mg of total protein lysate per IP assay as an optimal starting point.

Protein yield varies widely for any given cell line, and it is strongly recommended to perform a "test plate" protein quantitation, particularly if you are unsure of the expected protein yield from your experimental conditions. This is a simple procedure and is performed as follows;

Cell lysate preparation with BlastR™ Lysis System

1. Grow and treat cells as required (The volumes used in this protocol assume the use of a 150 cm^2 plate).
2. Remove culture media and wash the cells twice with 10 ml of room temp PBS. Note: remove as much PBS as possible prior to adding BlastR™ lysis buffer in order to maximize cell lysis.
3. Add 300 µl of BlastR™ lysis buffer (supplemented with 3 µl of 100X protease inhibitor cocktail) and lyse cells using a cell scraper. The lysate will become highly viscous due to nuclear lysis.
4. Use a 1 ml pipette to transfer the crude lysate into a BlastR™ filter that is in a 15 ml collection tube (see Appendix VII). Note: using a cut pipette tip may aide in transferring the viscous lysate.
5. Use a supplied filter plunger to compress the BlastR™ filter and collect the lysate flow through, including any bubbles that may be eluted at the end of the compression step, into a clean tube (see Appendix VII).
6. Optional: Centrifuge the lysate at approximately 10,000 g for 1 minute at 4°C. Transfer to a new tube and quantitate the lysate protein concentration as described below.

Protein Quantitation for BlastR™ Cell Lysate

1. Aliquot 20 µl of cell lysate into a microfuge tube.
2. Dilute lysate with 80 µl of BlastR™ Dilution Buffer and mix well.
3. Make a Blanking Buffer by adding 20 µl of BlastR™ Lysis Buffer to 80 µl of BlastR™ Dilution Buffer.
4. Aliquot 1 ml of Precision Red™ Advanced Protein Assay Reagent into each of two 1ml cuvettes.
5. Add 20 µl of diluted cell lysate to one cuvette and 20 µl of the Blanking Buffer to the second cuvette.
6. Cap the cuvettes and mix well by inverting several times.
7. Incubate the samples for 1 minute at room temperature.
8. Blank the spectrophotometer against the Blanking Buffer sample at 600 nm wavelength.

9. Read the lysate sample at 600 nm.

10. Use the equation below to determine the lysate protein concentration.

sample reading OD_{600} x 25 = protein concentration in mg/ml

NOTE: *readings below 0.05 or above 0.5 are close to the linear range capacity of the protein assay. For readings >0.5 samples can be diluted. For readings <0.05 more lysate can be added to the ADV02 (up to 50 µl). See Table 6 for multipliers to convert spectrophotometer readings to mg/ml lysate protein concentration.*

Table 6: Multiplier values for protein concentration measurements of lysates

Calculating Total Protein Concentration Per Plate

Volume of diluted cell lysate added to 1 ml of Precision Red Protein Assay reagent (µl)	Multiplier to use with sample reading OD_{600}
10	50
20	25
30	16.5
40	12.5
50	10

1. Multiply protein concentration (mg/ml) by 0.3 ml (protein volume) to determine test plate protein content.

   Protein concentration x volume = Test plate protein content (mg)

2. If the test plate protein content is > 2 mg then a higher volume of lysis buffer should be used (see Table 2). NOTE: it is important to maintain the recommended ratio between Lysis and Dilution buffers as the final buffer composition of the lysate will influence the stringency of PTM enrichment.

3. If there is insufficient protein in one plate, it is recommended to use 2 or more plates per IP. In this case plates will be harvested in series, transferring the original 300 µl of Lysis Buffer between plates.

4. When performing the Assay Protocol STEP 1, the protein concentration readout (mg/mL) will be lower due to dilution of lysate with dilution buffer prior to measuring the protein concentration; however, total protein per plate will remain the same.

VI: Appendix VI: Lysis and Dilution Buffer Prep

Supplement the required volume of BlastR™ Lysis Buffer and BlastR™ Dilution Buffer with de-SUMOylase inhibitor (Part # NEM09BB) and protease inhibitor cocktail (Cat # PIC02). Remember you will require approximately 4X the volume of Dilution Buffer to Lysis Buffer. Recipes for 1 ml of Lysis Buffer and 4 ml of Dilution Buffer are given below. Final required volumes will need to be determined by the end user.

Supplemented BlastR™ Lysis Buffer (1.0 ml)

BlastR™ Lysis Buffer	980 µl
De-SUMOylase Inhibitor (100X)	10 µl
Protease Inhibitor cocktail (100X)	10 µl

Table 7: Calculations for BlastR™ Lysis Buffer

1.0 ml	2.0 ml	5.0 ml	10.0 ml
980 µl	1960 µl	4.9 ml	9.8 ml
10 µl	20 µl	50 µl	100 µl
10 µl	20 µl	50 µl	100 µl

Supplemented BlastR™ Dilution Buffer (4.0 ml)

BlastR™ Dilution Buffer	3,920 µl
De-SUMOylase Inhibitor (100X)	40µl
Protease Inhibitor cocktail (100X)	40µl

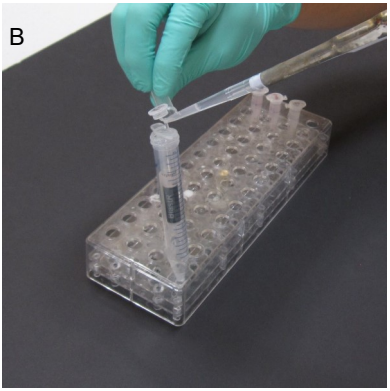
Table 8: Calculations for BlastR™ Dilution Buffer

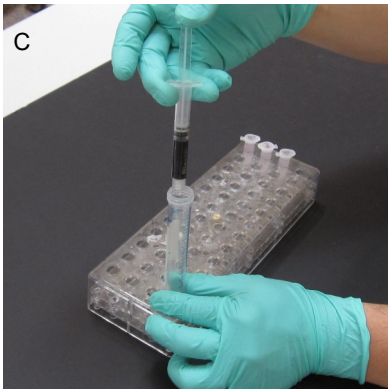
4.0 ml	8.0 ml	20.0 ml	40.0 ml
3.92 ml	7.84 ml	19.6 ml	39.2 ml
40 µl	80 µl	200 µl	400 µl
40 µl	80 µl	200 µl	400 µl

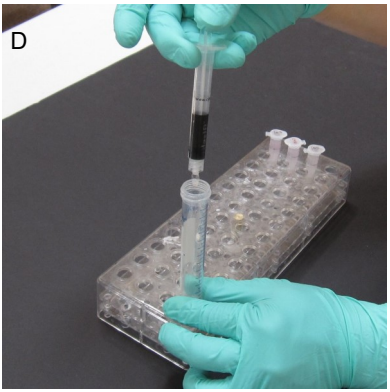
Limited Use Statement

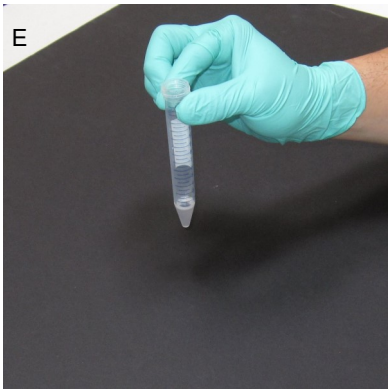
Figure 5: Filtering genomic DNA from cell lysate

Cytoskeleton's proprietary filtering system provides end-users with a very fast and simple method to remove genomic DNA from viscous cell lysates. This page shows example pictures that depicts utilization of the filter system.









A: BlastR™ Lysate Filter

B: Lysate is loaded into the filter that was placed in a15ml tube

C: Plunger is placed into the syringe and lysate is passed through the filter by compression

D: Collect lysate, including bubbles through complete compression

E: Filtered lysate

Protein Quantitation Assay

1. Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Part # GL50) to each of two 1ml cuvettes.
2. Mix 10 µl of BlastR™ Lysis Buffer and 40 µl of BlastR™ Dilution Buffer to a clean tube on ice. This will be used for the protein reading blank sample.
3. Add 20 µl of the Lysis/Dilution buffer mix (from step 2) to the first cuvette and mix by inverting two to three times.
4. Add 20 µl of the diluted cell lysate (from experiment) to the second cuvette, mix as above.
5. Incubate samples for 1 min at room temperature.
6. Blank spectrophotometer with the Lysis/Dilution buffer mix sample (from step 3).
7. Measure absorbance of the lysate sample (from step 4) at 600 nm.
8. Determine the lysate protein concentration as follows;

sample reading OD_{600} x 5 = protein concentration in mg/ml

NOTE: *readings below 0.05 or above 0.5 are close to the linear range capacity of the protein assay. For readings >0.5 samples can be diluted. For readings <0.05 more lysate can be added to the ADV02 (up to 50 µl). See Table 9 below for multipliers to convert spectrophotometer readings to mg/ml lysate protein concentration.*

9. If there is insufficient protein in one plate, it is recommended to use 2 or more plates per IP. In this case plates will be harvested in series, transferring the original 300 µl of Lysis Buffer between plates.

Table 9: Multipliers to Convert Spectrophotometer Readings to mg/ml Lysate

Volume of cell lysate added to 1 ml of Precision Red Protein Assay reagent (µl)	Multiplier to use with sample reading OD_{600}
10	10
20	5
30	3.3
40	2.5
50	2.0

VI: Appendix IX: Recommended Control Reactions

There are several control reactions that are recommended as part of this assay;

A) SUMO-2/3 IP Control Bead

IP Control Beads (Cat# CIG01-Beads) are included in the kit and are used to determine the amount of protein-of-interest that may be binding non-specifically to the beads. The SUMO-2/3 IP Control Beads contained in this kit are sufficient to carry out 10 IP reactions. Instructions for use are given in the assay protocol (STEP 3).

In some cases unmodified proteins of interest binds to Control Beads non-specifically. It is very rare that SUMO-2/3 modified species bind non-specifically and as these species run at a higher molecular weight than unmodified protein the issue of non-specific binding is not a common issue. In some cases, however, non-specific binding of SUMO-2/3 modified species is an issue. If this is the case then Control Beads can be used to pre-clarify the lysate and this step may improve the specific signal window. Control Beads can be purchased separately in cases where they are needed for pre-clarification of lysates.

B) Total SUMOylated species

After probing the IP reactions with an antibody to the protein of interest it is recommended to re-probe the blot with an anti-SUMO-2/3 antibody. This control reaction allows the end-user to confirm that the SUMO-2/3 IP reaction has enriched for total SUMOylated species in the lysate. An HRP-conjugated anti-SUMO-2/3 antibody is included in this kit (Cat# ASM23-HRP-S) and instructions for use are given in the Western Blot protocol (STEP 4B). An example of total SUMOylated protein detection after IP is given in Fig. 3A in the appendix.

C) Input

Include a sample of the original pre-IP lysate on the western blot. We recommend 0.5-2% of IP lysate volume. This serves as a marker for the unmodified protein band.

D) Inhibitor negative sample

De-SUMOylation will occur very rapidly in the absence of the inhibitor (Part# NEM09BB). In cases where the SUMOylation 2/3 signal is very weak or the primary detection antibody gives spurious bands in a western, the inclusion of a lysate that is processed in the absence of inhibitor will often confirm the authenticity of the SUMOylated 2/3 species.

Lysis and Dilution Buffer Prep

Signal-Seeker™ PTM affinity beads have been optimized to minimize light-chain leaching; however, interference remains a possibility. If you experience interference when examining modified proteins with mouse mAbs we highly recommend using a 1:1000 dilution of an HRP-conjugated secondary antibody that recognizes intact, native mouse primary antibodies. (e.g. anti-mouse-HRP TrueBlot ULTRA antibody from Rockland, Cat. # 18-8817-30).

Endogenous detection of β-catenin are reported below in Fig 6, and was generated using the Signal-Seeker™ Acetyl-Lysine Detection Kit. The data shows results obtained using various secondary reagents. Notice that a traditional anti-mouse-HRP secondary may detect light chain, and antibody-protein G high molecular weight complexes (Fig 6A). Secondary reagents that only detect intact, native mouse antibodies will not detect these interfering bands; however, there is a noticeable difference in signal intensity between these types of secondary reagents (Fig 6B-D).

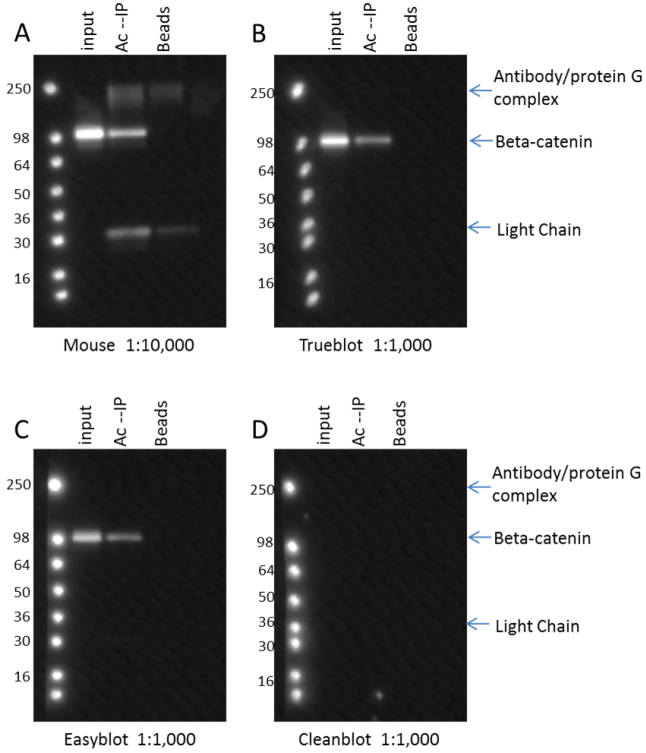


Fig 6: Use of alternative secondary reagents to detect mouse primary antibodies. Lysate from untreated A431 cells were isolated using BlastR lysis system. IP was performed using AAC04 beads (60μg) (Ac-IP). Total cell lysate (Input) and immunoprecipitated samples were separated by SDS-PAGE and analyzed by western blot with β-catenin mouse monoclonal antibody (BD Biosciences, 1:2000). (A) Anti-mouse HRP 1:10,000, (B) TrueBlot Ultra (Rockland, 1:1000), (C.) EasyBlot (Genetex, 1:1000), or (D) CleanBlot (ThermoFisher, 1:1000) secondary reagents were used to detect the protein.

VII: Changes Made from previous manual version

1) No changes made from previous version

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