Subject: Comparison of the BlastR Rapid Lysate Prep Kit with conventional lysis buffers

Clone: na

Catalog #: BLR01, BLR02

Description: Utilization of BlastR Lysate Prep kit to obtain a complete protein profile from cell culture or tissue samples

Isolating proteins from all cellular compartments can be achieved using denaturing lysis buffers; however, denaturing buffers have distinct disadvantages compared to non-denaturing lysis buffers, such as their incompatibility with standard colorimetric protein assays, copious viscosity due to genomic DNA contamination, and a negative effect on downstream immunoprecipitation assays. Here we describe Cytoskeleton's newly developed BlastR™ Rapid Lysate Prep kit that utilizes a denaturing buffer, BlastR™ lysis buffer, to isolate proteins from all cellular compartments resulting in a protein profile similar to other denaturing buffers like Laemmli. Unlike established denaturing buffers, BlastR™ buffer is compatible with conventional protein assays for easy quantitation. Additionally, the BlastR™ filter, included in this kit, removes genomic DNA contamination much more rapidly, gently and effectively than sonication or needle shearing. This system was developed to be compatible with immunoprecipitation reagents; specifically, immunoprecipitation of post-translational modifications. The BlastR™ rapid lysate prep kit can be used on cell culture or tissue to obtain lysates for use in downstream western and immunoprecipitation assays. This newly developed BlastR™ lysate prep kit provides the user all of the advantages of using a denaturing buffer, but with the ease and simplicity of using a standard non-denaturing buffer.

Introduction

The utilization of buffers like RIPA or NP-40 are commonly used to lyse cells for protein analysis by western blot or immunoprecipitation. Unfortunately, there are situations where these buffers are inadequate, such as when investigating membrane bound, DNA associated nuclear proteins, and other difficult proteins (1). Obtaining an incomplete protein profile with RIPA or NP-40 may result in an inadvertent misrepresentation of the results. In these situations a denaturing lysis buffer may be preferred as it is significantly better at isolating proteins from all cellular compartments and obtaining a more complete protein profile (1).

Denaturing buffers are also beneficial due to their ability to denature some proteases as well as proteins that alter protein modifications like desumoylases (2). Although denaturing buffers have key advantages over non-denaturing buffers they are aren't commonly used because of their significant drawbacks, such as incompatibility with conventional protein assays, significant genomic DNA contamination, and disruption to immunoprecipitation reagents (3). These drawbacks can result in extended preparation time and artificial damage to target proteins in the case of shearing genomic DNA (3).

Here we describe a newly developed denaturing buffer system, termed BlastR Rapid Lysate Prep, which provides all the benefits of utilizing a denaturing lysis buffer while eliminating the drawbacks. Importantly, the proprietary buffer formulation and BlastR™ filter enable conventional protein measurements, removes genomic DNA contamination, and is compatible with immunoprecipitation reagents to enable quantitative investigation of any target protein.

Results & Discussion

Isolation of proteins from cellular organelles with BlastR lysis buffer and other established lysis buffers

All lysis buffers have pros and cons and choosing the right lysis buffer is essential to obtain meaningful results; and is often highly dependent on the downstream application (1). For example, when active protein is necessary, then utilizing a denaturing buffer is not appropriate. Conversely, if obtaining a complete profile of proteins is the objective, then using a lysis buffer that can not effectively isolate proteins from specific organelles will result in an incomplete profile, and may likely skew interpretation. To highlight the ability of various buffers to capture proteins from all cellular compartments,

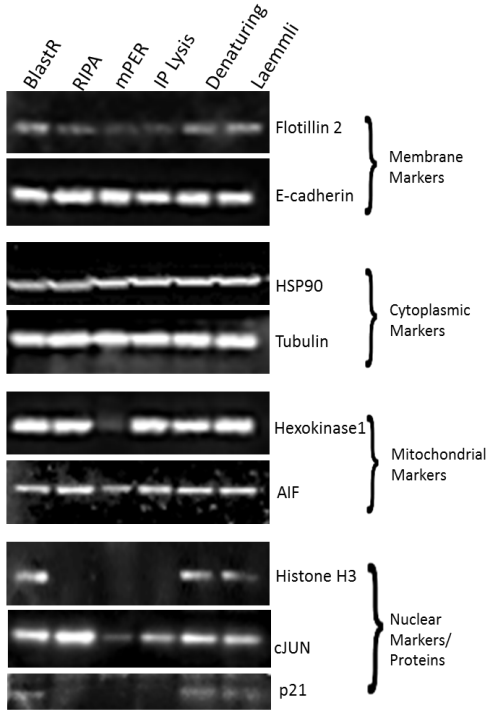

Figure 1. ***Comparison of BlastR lysis buffer to alternative lysis buffers.*** A431 cells were lysed with BlastR, RIPA, mPER, IP lysis, Denaturing (1% SDS), and Laemmli lysis buffers. All denaturing lysates had genomic DNA removed using BlastR filter. Isolation of proteins from the membrane, cytoplasmic, mitochondrial, and nuclear markers were determined using antibodies against the respective compartment marker proteins.

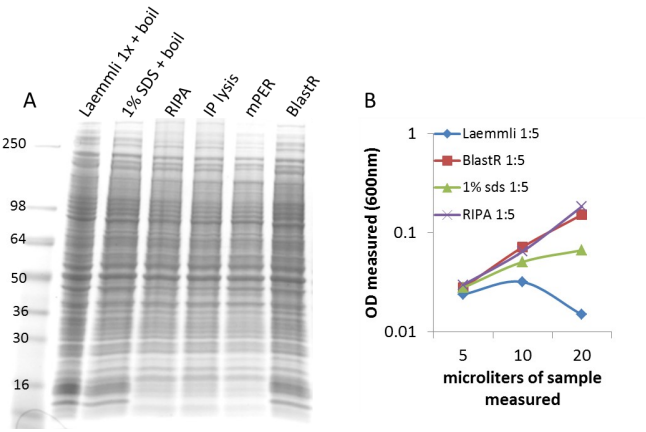

Figure 2. ***BlastR lysis buffer characteristics.*** (A) A431 cells were lysed with BlastR, RIPA, mPER, IP lysis, Denaturing (1% SDS), and Laemmli lysis buffers. All denaturing lysates had genomic DNA removed using the BlastR filter. Coomassie stain was performed to obtain a protein isolation profile with these buffers. (B) Protein quantitation of RIPA, BlastR, 1% SDS, and Laemmli was performed using a standard colorimetric assay (ADV02). A titration of 5, 10, and 20 µL was performed to determine the accuracy of protein measurement for each lysis buffer.

A431 cells were lysed with BlastR™, RIPA, mPER (Thermofisher, MA), IP Lysis (Pierce,), Denaturing (1% SDS), and Laemmli lysis buffers. Data from figure 1 shows that the BlastR™ buffer effectively isolates protein from all cellular compartments similar to other denaturing buffers like Laemmli and 1% SDS buffers. RIPA and less stringent buffers like mPER and IP lysis buffer, as expected, were ineffective at isolating some protein markers from the membrane and nuclear fractions, which have been shown previously in the literature. Denaturing lysis buffers are the optimal buffers to use when trying to obtain a complete profile of changes to a target protein.

Unique characteristics of BlastR buffer

When studying changes in cytoplasmic, nuclear or membrane proteins in response to stimuli, where equal protein loading between samples is paramount, the existing repertoire of lysis buffers may be inadequate. Data in figure 1 showed that BlastR™ buffer behaves similarly to denaturing buffers in its ability to isolate proteins from all cellular compartments. Data in figure 2A showed a similar result when comparing global protein profiles, which was visualized using coomassie staining. The coomassie staining for BlastR™ is similar to Laemmli and 1% SDS denaturing buffer, while the staining for RIPA, mPER, and IP lysis are less intense suggesting that there is less protein isolated with non-denaturing buffers.

One unique feature of BlastR™, distinct from other denaturing lysis buffers, is the ability to obtain accurate protein concentrations with conventional colorimetric assays. Titrations of BlastR™, RIPA, Laemmli, and 1% SDS lysates were measured using ADV02 protein assay, which is a standard colorimetric assay similar to Bradford (Figure2B). Note that Laemmli and 1% SDS buffer protein readings do not increase in a linear fashion. This inaccurate reading for denaturing buffers is often cause by high levels of detergents or reducing agents. In contrast, both BlastR™ and RIPA protein measurements increase linearly; for example, when twice as much protein is measured, the OD reading doubled as expected.

Equal concentrations of RIPA, IP lysis, mPER, and BlastR™ buffer were loaded in figure 2A; however, because Laemmli and 1% SDS lysates can not accurately be measured using conventional colorimetric assays, equal volumes were loaded relative to BlastR™ buffer. Alternative methods can be used to measure denaturing lysate protein concentrations such as specialized protein assays that require extra steps and reagents. Additionally, measuring proteins with a nanodrop machine is possible; however, the manufacturer notes in a technical bulletin that detergents in the lysis buffer can cause the pedestal stage to become “unconditioned”. These data highlight the utility in having a buffer that isolates proteins like a denaturing buffer, but is simple to use like a non-denaturing buffer.

Efficient removal of genomic DNA contamination using BlastR™ Filters

A significant hurdle when working with denaturing buffers is the ability to effectively remove genomic DNA contamination (3), which can interfere with protein measurements using colorimetric and nanodrop methods, migration of proteins in an SDS acrylamide gel, and can prevent protein and affinity matrix interaction during immunoprecipitation assays. The conventional methodology to reduce viscosity is to shear the DNA by using a syringe needle or sonicating the sample. Both of these methods shear but do not remove the genomic DNA, which can still interfere with downstream applications. Additionally, both methods can significantly affect protein integrity. An alternative method is DNA digestion using a DNAse; however, this also requires extra time and optimization.

The BlastR™ filter is a proprietary tool that can effectively remove genomic DNA contamination in under 1 minute. Figure 3A shows genomic DNA contamination in A431 cell lysate after treatment with the BlastR™ filter, syringe needle, or sonication. There is almost complete removal of genomic DNA, which is not the case using conventional syringe needle or sonication. Importantly, genomic DNA contamination can significantly affect total protein migration through an SDS– acrylamide gel (Figure 3B). Altered migration caused by genomic DNA contamination can significantly affect interpretation of western blots; for example, the smeared EGFR pattern seen in the unfiltered lysate may be interpreted as increased expression relative to the filtered sample (Figure 3C). These data highlight the need to effectively remove genomic DNA contamination when using denaturing buffers, and BlastR™ filters

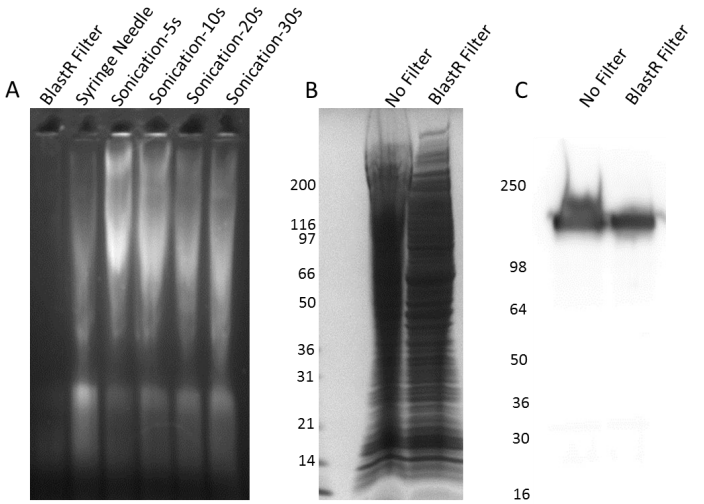

Figure 3. ***BlastR lysis filter is effective at removing genomic DNA.*** (A) A431 cells were lysed with a denaturing lysis buffer. Genomic DNA was removed or sheared with BlastR filter, syringe needle or sonication for 5, 10, 20, and 30 seconds. 2% of lysate was analyzed by ethidium bromide, agarose gel electrophoresis. (B) Lysate from A431 cells lysed with a denaturing buffer was either unfiltered or filter with the BlastR filter. Sample were separated with SDS-PAGE and visualized using Coomassie stain. (C) Duplicate samples from B were separated by SDS-PAGE, transferred to PVDE, and EGFR protein was examined using an EGFR antibody.

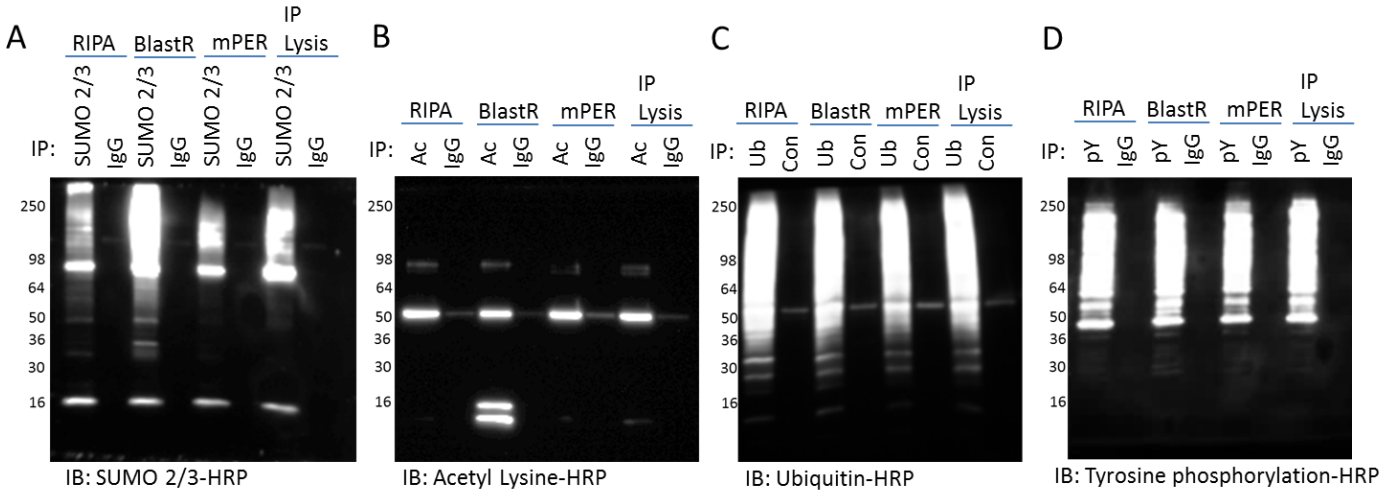

Figure 4. ***BlastR lysis system effective for immunoprecipitation of an array of PTM profiles.*** (A) A431 cell lysate made with RIPA, BlastR, mPER, or IPLysis was immunoprecipitated with SUMOylated 2/3 affinity beads or control beads. Total SUMOylated 2/3 profile was detected with a SUMO 2/3-HRP antibody. (B) A431 cell lysate made with RIPA, BlastR, mPER, or IPLysis was immunoprecipitated with Acetyl Lysine affinity beads or control beads. Total Acetylation profile was detected with an Acetyl Lysine antibody. (C) A431 cell lysate made with RIPA, BlastR, mPER, or IPLysis was immunoprecipitated with Ubiquitination affinity beads or control beads. Total Ubiquitinated profile was detected with a Ubiquitin-HRP antibody. (D) A431 cell lysate made with RIPA, BlastR, mPER, or IPLysis was immunoprecipitated with Phosphotyrosine affinity beads or control beads. Total tyrosyl phosphorylation profile was detected with a phosphotyrosine antibody.

provide an effective means to achieving this goal.

BlastR buffer can be used with immunoprecipitation reagents

A significant drawback of denaturing buffers is there incompatibility with immunoprecipitation affinity reagents. This problem may be due to the fact that these affinity reagents are comprised of antibody or protein binding domains, and when exposed to denaturing buffers can become unfolded. When blastR™ buffer is diluted according to the manufacturer's guidelines it can effectively be used with affinity matrix reagents for immunoprecipitation. Importantly, BlastR™ buffer should not be used for co-immunoprecipitation as its denaturing capabilities will disrupt the majority of protein:protein interactions.

In particular, BlastR™ buffer was developed for use with immunoprecipitation assays to capture low abundance PTM modificaitons for any target protein. Data from figure 4 highlights the utility of this buffer to effectively isolate a robust profile for SUMOylation 2/3, acetylation, ubiquitination, and tyrosine phosphorylation. Note that denaturing buffers are not effective at capturing a robust SUMOylation 2/3 profile potentially because of de-SUMOylase activity. Additionally, the highly acetylated histones are effectively captured from the BlastR™ buffer, but not the non-denaturing buffers because they are not as effective at isolating all nuclear proteins. Importantly, both ubiquitination and tyrosine phosphorylation profiles are as strong or better with BlastR™ buffer, and enrichment of these modifications are normally performed in non-denaturing buffers. The ability to use BlastR™ buffer to study all 4 PTMs in a single lysis system is unique from any available lysis buffer currently available, and may be the most effective system for studying crosstalk between these PTMs.

BlastR rapid lysate system effectively isolates protein lysates from tissue

To test the ability of the BlastR™ rapid lysate prep kit to effectively isolate protein lysates from tissue samples, brain, liver, and heart mouse tissue was obtain and lysed with BlastR™ lysis buffer. Tissue samples in BlastR™ buffer were homogenized and resulted in a viscous lysate. The viscous lysate was applied to the BlastR™ filter, and spun to remove excess cell debris. Samples were separated by SDS-PAGE and analyzed by coomassie (Figure 5A). Distinct protein patterns were present in the liver, brain, and heart tissue lysate. Duplicate samples were separated by SDS-PAGE, transferred to PVDF, and probed with a anti acetyl-lysine antibody (Figure 5B). Distinct acetylation patterns emerged for each tissue lysate. To determine if immunoprecipitation was possible, liver tissue lysate was immunoprecipitated with acetyl lysine affinity beads. Samples were then separated by SDS-PAGE, transferred to PVDF, and probed with an anti acetyl-lysine antibody (Figure 5C). These data provide evidence that the BlastR™ lysis system is effective for isolating proteins from tissue samples, which can be used for downstream western and immunoprecipitation applications; furthermore, this system may be particularly useful for studying post-translational modifications that may be occurring in different tissues.

Materials and Methods

Cell Culture and Reagents

A431 cells were grown in DMEM media (ATCC, VA) supplemented with 10% FBS (Atlas Biologicals, CO) and penicillin/streptomycin (ThermoFisher, MA). Trypsin/EDTA was obtained from Gibco (ThermoFisher, MA). Unless otherwise noted, chemicals were obtained from Sigma Chemical Co. (Sigma, MO). A431 cells were grown in 15cm dishes (Corning, NY).

Cell lysis and protein quantitation analysis

A431 cells were lysed with ice-cold BlastR lysis (Cytoskeleton, CO), RIPA, mPER (ThermoFisher, MA), IP lysis (ThermoFisher, MA), Laemmli, or 1% SDS denaturing buffer. All lysis buffers were supplemented with a cocktail of NEM, TSA, Na_3VO_4, and protease inhibitors (PIC02) (Cytoskeleton, CO). Laemmli and 1% denaturing lysates were boiled for 5min at 100 ^{0}C to adhere to standard protocol preparation. DNA was removed from all BlastR, 1% SDS, and Laemmli denaturing lysates by passing the lysate through the BlastR filter system (Cytoskeleton, CO). Alternative methods of genomic DNA shearing include needle syringe, or sonication as indicated.

RIPA, BlastR, Laemmli, and 1% SDS denaturing lysates were diluted at 1 part lysate to 4 parts BlastR dilution buffer. Protein concentrations were determined using a standard colorimetric protein assay, ADV02 (Cytoskeleton, CO), and measured at 600nm OD. 5, 10, and 20 μL of each lysate was measured.

For tissue lysate preparation, mouse liver, brain, or heart was lysed at 1 mL of BlastR lysis buffer per 100mg of tissue. For

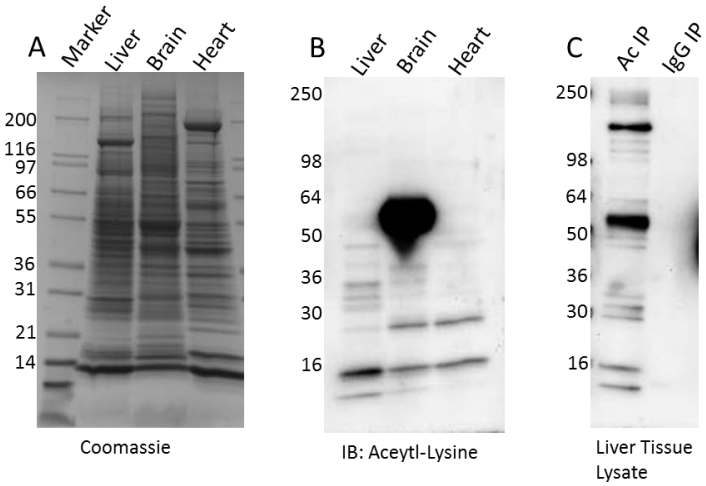

Figure 5. ***The BlastR lysis system is an effective tool for tissue lysate isolation.*** (A) BlastR lysis buffer was added to fresh mouse liver, brain, and heart tissue at 1mL of lysis buffer per 100 mgs of tissue. Tissue samples were then processed (please see method section). Lysate samples were separated using SDS-PAGE and visualized using coomassie stain. (B) Samples from A were run in duplicate and after separation by SDS-PAGE were transferred to PVDF. Total acetylation was examined using an Acetyl Lysine-HRP antibody. (C) Liver tissue lysate prepared in A was immunoprecipitated using Acetyl Lysine affinity beads. Samples were then separated by SDS-PAGE and transferred to PVDF. The total immunoprecipitated acetylation was examined using an Acetyl Lysine-HRP anti-

these experiments 100mg of tissue was processed. After addition of 1mL of BlastR™ lysis buffer to 100mg of fresh tissue, the sample was placed into a homogenizer and 10-12 strokes were applied. Tissue lysate became noticeably viscous. The lysate was then passed through the BlastR™ filter to remove genomic DNA. Equal volume of BlastR™ dilution buffer was then added to the filtered lysate and gently mixed. Lysate was then spun in a microcentrifuge at 14k rpm for 15min at 4°C to pellet any remaining tissue debris. Additional BlastR™ dilution buffer was added to the recovered supernatant for a final dilution of 1:5. Protein concentration was determined using
ADV02.

Western immunoblot and coomassie assay
Lysates produced from BlastR, RIPA, mPER, IP lysis, 1% SDS denaturing, and Laemmli were separated using Tris-glycine SDS-polyacrylamide gel electrophoresis (ThermoFisher, MA). Polyacrylamide Gels were then stained with coomassie to visualize the total protein profile obtained using each type of lysis buffer. For tissue samples. Mouse liver, brain, and heart tissue lysed with BlastR buffer were separated using Tris-glycine SDS-polyacrylamide gel electrophoresis (ThermoFisher, MA). Polyacrylamide Gels were then stained with coomassie to visualize the total protein profile obtained from each tissue type.

Lysates produced from BlastR, RIPA, mPER, IP lysis, 1% SDS denaturing, and Laemmli were separated using Tris-glycine SDS-polyacrylamide gel electrophoresis (ThermoFisher, MA). and transferred to Immobilon- P membranes (Millipore, MA). Membranes were blocked for 1 hr at room temperature in Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 0.05% Tween-20 (TTBS) and 5% milk (Thrive Life, UT), and then incubated with TTBS (+/- 5% milk) solution containing the primary antibody for 1 hour at room temperature (RT). Membranes were washed in TTBS 3x10 minutes, prior to secondary Mouse or Rabbit (Jackson ImmunoResearch Laboratories, PA) antibody for 1hr at RT. Bound antibodies were visualized with horseradish peroxidase-coupled secondary antibodies and chemiluminescent reagent (Cytoskeleton, CO) according to the manufacturer's directions. Antibodies used: Ubiquitin-HRP (Cytoskeleton, CO), Phosphotyrosine-HRP (Cytoskeleton, CO), Acetyl Lysine-HRP (Cytoskeleton, CO), SUMO 2/3-HRP (Cytoskeleton, CO), tubulin (Cytoskeleton, CO), Flotillin -2 (Abcam, MA), E-cadherin (Abcam, MA), HSP90 (Abcam, MA), Hexokinase 1 (Abcam, MA), AIF (Abcam, MA), Histone H3 (Abcam, MA), cJUN (ThermoFisher, MA), p21 (Abcam, MA), HRP-anti-mouse secondary (Cytoskeleton, CO), HRP-anti- sheep secondary (Cytoskeleton, CO), and HRP-anti- rabbit secondary (Jackson ImmunoResearch, PA).

Co-immunoprecipitation assay
1 mg of sample lysate was immunoprecipitated with the appropriate amount of pY (APY03), Ub (UBA01), SUMO 2/3 (ASM24), Acetyl lysine beads, IgG beads (CIG01), or Ub control beads (CUB01). Samples were immunoprecipitated for 1-2 hr at 4°C on an end-over-end tumbler. After incubation, the affinity beads from each sample were pelleted, and washed 3X with BlastR™ wash buffer. Bound proteins were eluted using bead elution buffer (Cytoskeleton, CO) and detected by western immunoblotting.

References

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