V. 7.0

Signal-Seeker™ Ubiquitin Enrichment Kit

30 Assays

Cat. # BK161

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com

Manual Contents

I: Introduction: 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 individualy 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 a rapidly growing area in protein regulation studies (1-4). Table 1 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 1: Examples of PTM Cross-Talk in response to a given stimulus or physiological state

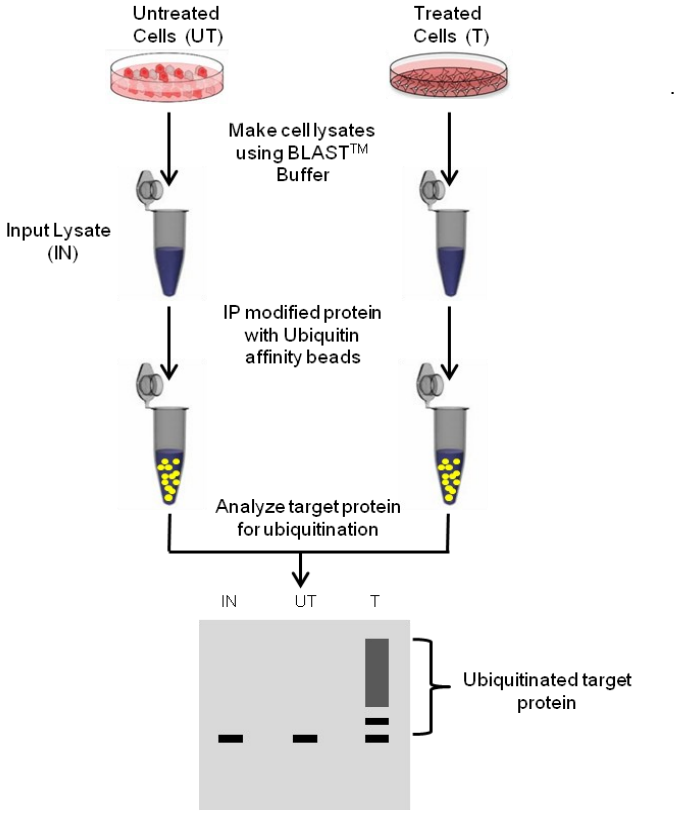
Protein	Co-dependent Modifications	Function	Ref.
Protein Kinase C (PKCα)	Phosphorylation SUMOylation Ubiquitination	**Observation:** PKCα is degraded over a 4h period following PKCa activation by the phorbol ester PMA. **Mechanism:** Mediated via a cascade of PTMs. A time-course for PMA treatment showed 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	Hyperphosphory-ation 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

I: Introduction: Assay Principle/Applications

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-Seekers™ are available for several key PTMs, including phosphorylation (phosphotyrosine), ubiquitination, SUMOylation and acetylation (see www.cytoskeleton.com for the full range of kits). 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



Applications

- Discover and publish novel regulatory mechanisms.
- Detect highly transient regulation of protein modifications.
- Confirm data generated from transfection or proteomic approaches
- Use different kits to build a temporal protein regulation profile.
- Investigate the role of known protein modifications in your system.
- Obtain data for endogenous proteins.
- Discover novel biomarkers.

I: Introduction: Assay Features

Assay Features

The study of endogenous PTMs poses sevaral technical challenges (see Table 2). 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 2 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 2: 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 freature 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 chemilluminescent 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™ Bufer system consisting of a Lysis buffer and a Dilution buffer. 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.

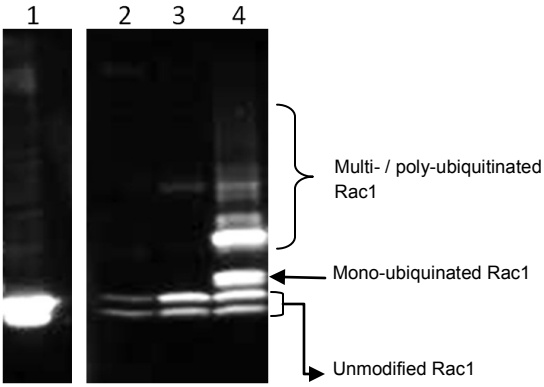
I: Introduction: Ubiquitin Affinity Beads

Signal Seeker™ Ubiquitin Affinity Beads

The Ubiquitin Affinity Beads (Ubiquitin Affinity Beads 1: Cat # UBA01) contain crosslinked Ubiquitin Binding Domains (UBDs), also called Ubiquitin-associated Domains (UBAs) and, in cases where multiple domains are expressed in a single protein, Tandem Ubiquitin Binding Entities (TUBES). Cytoskeleton scientists have developed a proprietary formulation of UBDs that have the unique characteristic of capturing both monoubiquitinated and polyubiquitinated proteins with high affinity. As mono-, multi– and poly-ubiquitination often confer unique, non-redundant properties to their target protein it is crucial to obtain the complete ubiquitin profile of any given target protein. Cytoskeletons Ubiquitin Affinity Beads are a powerful tool for the complete analysis of protein ubiquitination proflies.

Figure 2 shows the detection of endogenous mono– and poly-ubiquitinated Rac1 protein from 3T3 cells treated with the bacterial toxin CNF1. The data shown in figure 2 agrees with published data and demonstrates the utility of the Ubiquitin Affinity beads and Signal-Seeker™ kits in studying the rapidly growing area of small G-protein regulation by ubiquitin (5, 7-9).

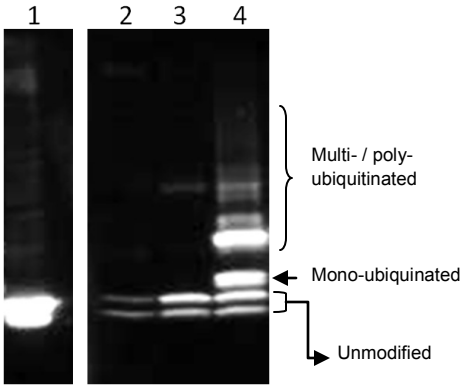
Figure 2: Ubiquitin Affinity Beads show efficient capture of both Mono- and Poly-ubiquitinated target proteins



Swiss 3T3 cells were either untreated (Lane 3) or treated (Lane 4) with bacterial toxin CNF1 for 3 hours prior to lysis in BLASTR™ buffer. Lysates (300 µg per assay) were treated as outlined in the Signal-Seeker™ Ubiquitin Enrichment manual. The western blot was probed with an anti-Rac1 antibody. CNF1 treatement resulted in detection of mono– and polyubiquitinated species of Rac1 which agrees with previous reports (5). Lane 1, 3% of input signal; Lane 2, control beads plus lysate. The protease inhibitor MG132 was included in all lysates (10 µM/3h). Clear ubiquitin signals were also detected in the absence of MG132 (data not shown).

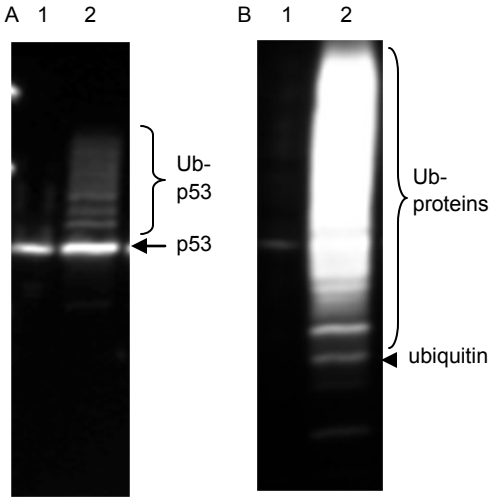
The data shown below was generated using the Signal-SeekerTM Ubiquitin Enrichment kit. The target proteins detected are from endogenous Rac1 and p53 respectively.

Figure 3: Detection of Ubiquitinated Rac1



Swiss 3T3 cells were either untreated (Lane 3) or treated (Lane 4) with bacterial toxin CNF1 for 3 hours prior to lysis in BLASTR™ buffer. Lysates (300 µg per assay) were treated as outlined in the Signal-Seeker™ Ubiquitin Enrichment manual. The western blot was probed with an anti-Rac1 antibody. CNF1 treatement resulted in detection of mono– and polyubiquitinated species of Rac1 which agrees with previous reports (5). Lane 1, 3% of input signal; Lane 2, control beads plus lysate. The protease inhibitor MG132 was included in all lysates (10 µM/3h).

Figure 4: Detection of Ubiquitinated p53 and total ubiquitinated proteins



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™ Ubiquitin Enrichment manual. The western blot (A) was probed with an anti-p53 antibody and re-probed (B) with anti-ubiquitin-HRP antibody. Lane A1, 2% input lysate; Lane A2, IP from 500 µl of HeLa lysate enriched using ubiquitin affinity beads. Ubiquitinated p53 is clearly visible in the IP lane which agrees with published data (6). Western blot (B) shows blot (A) re-probed with anti-ubiquitin-HRP antibody. Lane 1 shows a slight signal at the position of p53 as the blot was not stripped prior to re-probing.

II: Changes Made from previous manual version

Changes made from v6.0

1) The composition of BLASTR™ Wash Buffer has been altered to reduce wash stringency while maintaining target specificity. Specifically 0.1% SDS and 0.5% sodium deoxycholate have been removed from the Wash Buffer. All other components are identical between BLASTR™ Wash Buffer and BLASTR-2™ Wash Buffer.

III: Kit Contents

This kit contains enough reagents for 30 ubiquitin assays and 10 control assays. When properly stored, kit components are guaranteed stable for a minimum of 6 months.

Table 3: Kit Contents and storage prior to reconstitution of components

Reagents	Cat. # or Part # *	Quantity	Storage
BLASTR™ Lysis Buffer	Part# BLST01	1 bottle	4°C desiccated
BLASTR™ Dilution Buffer	Part# BDB01	1 bottle	4°C desiccated
BLASTR-2™ Wash Buffer	Part# BWB02	1 bottle	4°C desiccated
De-ubiquitination/SUMOylation Inhibitor (N-ethylmaleimide)	Part# NEM09	1 tube	4°C desiccated
Protease Inhibitor Cocktail	Cat# PIC02	1 tube	4°C desiccated
Ubiquitin Affinity Beads 1	Cat# UBA01-Beads	3 tubes	4°C desiccated
Ubiquitin IP Control Beads (to assess non-specific binding of target protein)	Cat# CUB01-Beads	2 tubes	4°C desiccated
Anti-Ubiquitin-HRP antibody	Cat# AUB01-HRP-S	1 tube	4°C desiccated
Precision Red™ Advanced Protein Assay Reagent	Part #GL50	1 bottle (100 ml)	4°C
Bead Elution Buffer	Part# BEB01	1 tube (1.3ml)	4°C
DMSO	Part# DMSO	2 tubes (1.5 ml each)	4°C
Chemiluminescent detection reagent A	Part # CLRA-10 ml	1 bottle	4°C
Chemiluminescent detection reagent B	Part# CLRB-10 ml	1 bottle	4°C
Spin columns	Part# SPN22	40 columns	4°C or room temp.
Spin column collection tubes	Part# SPN22-CT	40 tubes	4°C or room temp.

* Items with part numbers (Part #) are not sold separately and available only in kit format. Items with catalog numbers (Cat. #) are available separately.

The 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
- SDS-PAGE system and buffers
- Western transfer system and buffers
- Primary antibody to target protein
- HRP-labeled secondary antibody
- 2 mercaptoethanol
- Chemiluminescence documentation instrument
- Vortex

IV: Reconstitution and Storage of Components

Many of the kit components are provided in lyophilized form. Prior to beginning the assay, you will need to reconstitute several components as shown in Table 4. When properly stored and reconstituted, components are guaranteed stable for 6 months.

Table 4: Component Storage and Reconstitution

Kit Component	Reconstitution	Storage Conditions
BLASTR™ Lysis Buffer	Resuspend in 15 mls sterile water to give a 1X stock solution	4°C
BLASTR™ Dilution Buffer	Resuspend in 130 mls sterile water to give a 1X stock solution.	4°C
BLASTR-2™ Wash Buffer	Resuspend in 130 mls sterile water to give a 1X stock solution.	4°C
De-ubiquitiation/SUMOylation Inhibitor (1M N-ethylmaleimide)	1) Resuspend in 1 ml of DMSO (provided in kit) for a 100X stock solution. 2) Aliquot 10 x 100 ul volumes. This reduces freeze thaw cycles which can reduce inhibitor potency.	-20°C
Protease Inhibitor Cocktail	Resuspend in 1 ml of DMSO (provided in kit) for a 100X stock solution	-20°C
Ubiquitin Affinity Beads 1	Resuspens each tube in 420 µl of water. Each IP assay uses 40 µl of bead slurry.	4°C
Control beads for ubiquitin IP	Resuspens each tube in 220 µl of water. Each IP assay uses 40 µl of bead slurry.	4°C
Anti-Ubiquitin-HRP antibody	Resuspnd in 25 µl of water	4°C
Precision Red™ Advanced Protein Assay Reagent	Not required	Room temp.
Bead Elution Buffer	Not required	Room temp.
DMSO	Not required	Room temp.
Chemiluminescent detection reagent A	Not required	Room temp.
Chemiluminescent detection reagent B	Not required	Room temp.
Spin columns	Not required	Room temp.

STEP 1: Test Plate to Determine Protein

It is recommended to aim for 0.5-1.0 mg of total protein lysate per assay with 1.0 mg being an optimal starting point. Protein concentrations of between 0.5-1.2 mg/ml (post lysis & dilution) are recommended.

Protein yield varies widely in 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;

1. Grow a test plate using the cell line, growth confluency and treatment conditions that you will be using for your experimental protocol. The volumes used in this protocol assume the use of a 150 cm^2 plate. Table 5 gives suggested lysis and dilution volumes per 150 cm^2 plate for given cell densities.

Table 5: BLASTR™ Lysis/Dilution Buffer Chart

Recommended BLASTR™ Lysis Buffer volume	Recommended BLASTR ™ Dilution Buffer volume
300 µl	1.8 ml

2. Make up the following buffers as follows and place on ice. Volumes are per 150 cm^2 plate at high cell density.

   Supplemented BLASTR™ Lysis Buffer (0.3 ml)

BLASTR™ Lysis Buffer	294 µl
De-Ubiquitination Inhibitor	3 µl
Protease Inhibitor cocktail	3 µl

   Supplemented BLASTR™ Dilution Buffer (2.0 ml)

BLASTR™ Dilution Buffer	1.96 ml
De-Ubiquitination Inhibitor	20 µl
Protease Inhibitor cocktail	20 µl

3. Remove tissue culture plate from incubator and gently aspirate off growth media.

4. Wash cells **two times** in 10 ml each of 4°C PBS buffer pH 7.4.

5. Aspirate off PBS. After the final PBS wash tilt the plate and leave for 20-30 seconds to collect residual PBS. This is a critical step as residual PBS will dilute BLASTR-2™ Lysis Buffer and make it less effective at lysing cells.

6. Lyse cells by adding 300 µl of supplemented BLASTR™ Lysis Buffer (see Table 5 for recommended buffer volumes) to the plate and harvest using a cell scraper.

   NOTE: Cell lysate will become viscous during harvesting due to nuclear lysis and release of genomic DNA. Lysate transfer (below) may require a snipped pipette tip.

7. Transfer the lysate to a 2 ml tube (or similar) on ice. NOTE: at this point the total lysate volume should not exceed 2X the original lysate volume (e.g. 600 µl final volume for an original lysis volume of 300 µl).
8. Dilute the lysate to a total final volume of 2 ml (Table 5).
9. Vigorously shake the lysate for 10-20 seconds.
10. Vortex on high setting for 10 seconds. At this point there should be no viscocity in the lysate.
11. Shake the lysate for a further 5-10 seconds.
12. Centrifuge at 10,000 g (maximum speed in a microcentrifuge), 4°C for 10 minutes. A white pellet should be visible after centrifugation.
13. Transfer supernatants to fresh tubes and place on ice. Discard pellets.
14. Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Part # GL50) to each of two 1ml cuvettes.
15. Mix 10 ul each of supplemented BLASTR™ Lysis Buffer and supplemented BLASTR™ Dilution Buffer to a clean tube on ice. This will be used for the protein reading blank sample.
16. Add 10 µl of the supplemented Lysis/Dilution buffer to the first cuvette and mix by inverting two to three times.
17. Add 10 µl of diluted cell lysate to the second cuvette, mix as above.
18. Incubate samples for 1 min at room temperature.
19. Blank spectrophotometer with the supplemented Lysis/Dilution buffer mix.
20. Measure absorbance of the lysate sample at 600 nm.
21. Determine the lysate protein concentration as follows;

    sample reading OD_{600} x 10 = protein concentration in mg/ml

22. A protein concentration between 0.5 - 1.2 mg/ml indicates that there is sufficient protein in one 150 cm^2 plate to carry out 1-2 immunoprecipitations.
23. 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.

STEP 2: Recommended control Reactions

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

A) Ubiquitin IP Control Bead

Ubiquitin IP Control Beads (Cat# CUB01-Beads) are included in the kit and are used to determine the amount of non-specific bead binding to the protein of interest. The Ubiquitin 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 4).

In some cases Control Beads will bind non-specifically to the unmodified protein of interest. It is very rare that ubiquitin 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 ubiquitin 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 ubiquitinated species

After probing the ubiquitin IP reactions with an antibody to the protein of interest it is recommended to re-probe the blot with an anti-ubiquitin antibody. This control reaction allows the end-user to confirm that the ubiquitin IP reaction has enriched for total ubiquitin species in the lysate. An HRP-conjugated anti-ubiquitin antibody is included in this kit (Cat# AUB01-HRP-S) and instructions for use are given in the Western Blot protocol (STEP 5B).

C) Input

Include a sample of the original pre-IP lysate on the western blot. We recommend 2-5% of IP lysate volume. This serves as a marker for the unmodified protein band.

STEP 3: Treat Cells and Harvest Lysates

1. Treat tissue culture cells as required. Each IP assay requires approximately 0.5-1.0 mg of lysate protein.

2. If you do not know the approximate yield of total protein in your cell lysate we recommend that you use the procedure described in STEP 1 to determine the volume of lysis buffer required to yield a 1 mg/ml final diluted lysate concentration. Recommended volumes of BLASTR™ Lysis Buffer and BLASTR™ Dilution Buffer per 150 cm^2 tissue culture plate are given in Table 5.

3. Supplement the required volume of BLASTR™ Lysis Buffer and BLASTR™ Dilution buffer with de-ubiquitination inhibitor (10 µl per ml of buffer) and protease inhibitor cocktail (10 µl per ml of buffer). Remember you will require approximately 6X the volume of Dilution Buffer to Lysis Buffer (300 µl of Lysis buffer to 1.7 ml Dilution Buffer).

4. Place buffers on ice.

5. Obtain PBS pH 7.4 buffer (20 ml of PBS is required per tissue culture plate being processed), cell scrapers and liquid nitrogen (if you will be snap freezing cell lysates for later analysis. Snap freezing is highly recommended).

6. Before processing tissue culture cells, it is recommended to label tubes ready for lysate collection.

7. Remove tissue culture plate from incubator and gently aspirate off growth media.

8. Wash cells **two times** in 10 ml each of 4°C PBS buffer pH 7.4.

9. Aspirate off PBS. After the final PBS wash tilt the plate and leave for 20-30 seconds to collect residual PBS. This is a critical step as residual PBS will dilute BLASTR™ Lysis Buffer and make it less effective at lysing cells.

10. Lyse cells by adding the pre-determined volume of supplemented BLASTR™ Lysis Buffer (300 µl recommended starting point) to the plate and harvest using a cell scraper.

    NOTE: Cell lysate will become viscous during harvesting due to nuclear lysis and release of genomic DNA. Lysate transfer (below) may require a snipped pipette tip.

10. Transfer the lysate to a 2 ml tube (or similar) on ice. NOTE: at this point the total lysate volume should not exceed 2X the original lysate volume (e.g. 600 µl final volume for an original lysis volume of 300 µl).

11. Dilute the lysate by adding BLASTR™ Dilution Buffer according to the following calculation;

Original BLASTR™ lysis buffer volume x 6

Example: original lysis buffer volume of 300 µl would require 1.8 ml of BLASTR™ Dilution Buffer.

12. Vigorously shake the lysate for 10-20 seconds.
13. Vortex on high setting for 10 seconds. At this point there should be no viscocity in the lysate.
14. Shake the lysate for a further 5-10 seconds.
15. Centrifuge at 10,000 g, 4°C for 10 minutes. A white pellet should be visible after centrifugation.
16. Transfer supernatants to fresh tubes and place on ice. Discard pellets.
17. Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Part # GL50) to each of two 1ml cuvettes.
18. Mix 10 ul each of supplemented BLASTR™ Lysis Buffer and supplemented BLASTR™ Dilution Buffer to a clean tube on ice. This will be used for the protein reading blank sample.
19. Add 10 µl of the supplemented Lysis/Dilution buffer to the first cuvette and mix by inverting two to three times.
20. Add 10 µl of diluted cell lysate to the second cuvette, mix as above.
21. Incubate samples for 1 min at room temperature.
22. Blank spectrophotometer with the supplemented Lysis/Dilution buffer mix.
23. Measure absorbance of the lysate sample at 600 nm.
24. Determine the lysate protein concentration as follows;

    sample reading OD_{600} x 10 = protein concentration in mg/ml

25. A protein concentration between 0.5 - 1.2 mg/ml indicates that there is sufficient protein in one 150 cm^2 plate to carry out 1-2 immunoprecipitations.
26. 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 Lysis Buffer between plates (see Table 5).
27. Equalize lysate protein concentrations using a 1:1 dilution of BLASTR™ Lysis Buffer and BLASTR™ Dilution Buffer (inhibitors are not necessary for this step).
28. Lysates that will not be used straight away can be aliquoted and snap frozen in liquid nitrogen and stored at -70 to -80°C. Lysates should be stable for several months.

STEP 4: Immunoprecipitation (IP) Assay

1. Flick tube containing Ubiquitin Affinity Bead 1 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.
3. Flick tube containing Ubiquitin 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 for a control reaction to determine non-specific binding of ubiquitinated species.
5. Add lysate. We recommend 0.5-1.0 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.
6. Save a small amount of lysate (20 µl) to run as a western input lysate control.
7. Incubate the tubes on a rotating platform at 4°C for 2h.
8. Collect beads by centrifugation at 3-5,000 x g for 1 minute at 4°C.
9. Aspirate off as much supernatant as possible without disturbing the beads.
10. Wash beads in 1 ml BLASTR-2™ Wash Buffer (inhibitors are not necessary at this stage) for 5 minutes on a 4°C rotating platform.
11. Collect beads by centrifugation at 3-5,000 x g for 1 minute at 4°C.
12. Aspirate off as much supernatant as possible without disturbing the beads.
13. Repeat the wash step two more times.
14. After the final wash, completely remove buffer supernatant without disturbing the bead pellet. Optional Technical Tip: remove residual supernatant using a fine bore protein loading tip.
15. Add 30 µl of Bead Elution Buffer and resusend the beads by gently tapping/flicking the side of the tube. DO NOT use a pipette at this stage.
16. Incubate at room temperature for **exactly** 5 minutes.
17. 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.
18. 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.
19. 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.

20. Place samples in a boiling water bath for 5 minutes prior to running SDS-PAGE and western blot analysis, see STEP 5A & 5B.

STEP 5: Western Blot Protocol

STEP 5A: Western Blot for Identification of Protein of Interest

1. A primary antibody provided by the end user will be used for detection of the ubiquitinated version of the protein of interest. The SDS-PAGE and western blot should be performed according to your laboratory protocol.
2. 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 chemilluminescent reagent should be used in conjunction with an HRP-labeled secondary antibody capable of detecting your primary antibody. For mouse monoclonal antibodies (MAbs) we would recommend using a 1:20,000 dilution of an HRP-conjugated goat anti-mouse (eg. goat anti-mouse HRP conjugated IgG from Jackson Labs., Cat. # 115-035-068).
3. The chemilluminescent detection reagents supplied in this kit is sufficient for 10 minigel sized westerns. A volume of 2 ml of chemilluminescent 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 regaent 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.

STEP 5B: Determination of total ubiquitinated species in the IP

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

Method 1: Re-probe Blot with anti-ubiquitin-HRP

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

   NOTE: Stripping the blot prior to probing is not necessary as the signal from total ubiquitin 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-ubiquitin-HRP antibody diluted in TBST (no blocking agent) 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 regaent B (sufficient for one 8 x 7 cm membrane).
5. Add chemiluminescent reagent to membrane and incubate with gentle rocking at room temperature for 5 minutes prior to visualization of total ubiquitinated 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 ubiquitinated species IP will be very strong. It is therefore possible to keep a small volume (5 µl per IP) to run a separate western for the purpose of determining IP efficiency.

VI: Troubleshooting

Observation	Possible cause	Remedy
No target protein ubiquitination detected	There are several possible reasons for this result; 1) The protein of interest is not ubiquinated 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 ubiquitination 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 ubiquitinated species by using the anti-ubiquitin-HRP antibody to analyse the IP reactions and see below. 2) A timecourse is often appropriate particularly if signal transduction pathways are being analysed. Also a proteosome inhibitor such as MG132 (5-20 µM) can be added to the cell culture 2-3 hours prior to harvesting. MG132 treatment allows the accumulation of ubiquitinated species and increases chances of detecting low level, transient modifications. 3) It is important to make sure the primary detection 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.
Band visible at 55kD in all Ubiquitin Bead samples	The Ubiquitin Binding proteins used in this assay run at 55 kD. A small amount of protein can sometimes leach off the affinity beads and can occasionally be detected as a non-specific band by some primary or secondary antibodies.	Run a Ubiquitin Affinity Bead only sample that does not contain cell lysate. If the 55kD band is coming from the beads then it will be visible in this sample.
Unmodified protein band detected in IP	Ubiquitin Affinity Beads may detect a band that corresponds to the unmodified target protein. This may be non-specific or caused by de-ubiquitination during enrichment.	1) Make sure the de-ubiquitin 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) As ubiquitin modified protein will run at a higher molecular weight than unmodified protein (ubiquitin is 8 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. 3) Always run a Ubiquitn IP Control Bead reaction to determine degree of non-specific binding.
No pellet or flocculent pellet after cell lysis and	If lysis volumes different from those given in the method are used it is possible to get a flocculent pellet after centrifugation of lysates.	Follow recommended lysis method see table 5.

VII: References

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

2. Guo, Z, et al. 2012. Sequential posttranslational modifications program FEN1 degradation during cell cycle progression. *Mol. Cell.* **47**: 444-456.

3. Luo H-B, et al. 2014. SUMOylation at K340 inhibits Tau degradation through deregulating its phosphorylation and ubiquitination. *Proc. Natl. Acad. Sci. USA* **111**: 16586-16591.

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

5. Torrino S. et al. 2011. The E3 ubiquitin-ligase HACE1 catalyses the ubiquitination of active RAc1. Developmental *Cell* **21**: 959-965.

6. Jin L. et al. 2013. Epigallocatechin gallate promotes p53 accumulation and activity via the inhibition of MDM2-mediated p53 ubiquitination in human lung cancer cells. *Onc. Rep*. **29**: 1983-1990.

7. Nethe P. & Hordijk P. 2010. The role of ubiquitylation and degradation in RhoGTPase signaling. *J. Cell Sci.* **123**: 4011-4018.

8. Ding F. et al. 2011. Ubiquitination in Rho signaling. *Curr. Topics Med. Chem.* **11**: 2879-2887.

9. Visvikis O. et al. 2010. Direct modifications of Rho proteins: deconstructing GTPase regulation. *Biol. Cell* **102**: 377-389.

Limited Use Statement

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of product and components of product in research conducted by the buyer. The buyer cannot sell or otherwise transfer this product or any component thereof to a third party or otherwise use this product or its components for commercial purposes. Commercial purposes include, but are not limited to: use of the product or its components in manufacturing; use of the product or its components to provide a service; resale of the product or its components.

The terms of this Limited Use Statement apply to all buyers including academic and for-profit entities. If the purchaser is not willing to accept the conditions of this Limited Use Statement, Cytoskeleton Inc. is willing to accept return of the unused product with a full refund.

Some of the technology used in this kit has been licensed under Patent US 8,716,441 B2.

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com