



Signal-Seeker™ Phosphotyrosine Detection Kit

10 Assays

Cat. # BK160-S

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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

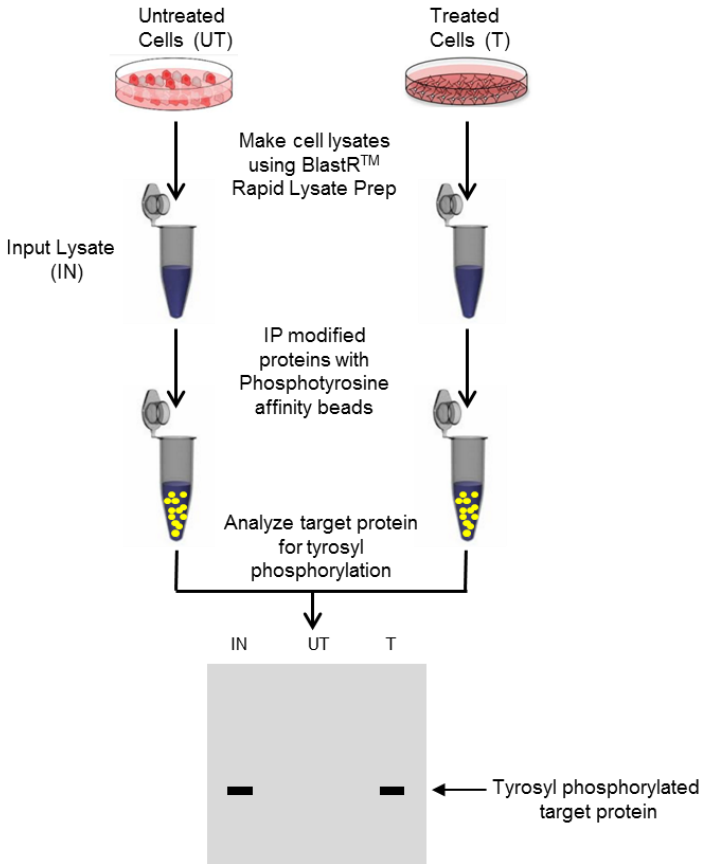
- Discover and publish novel regulatory mechanisms.
- Detect highly transient regulation of protein modifications.
- Confirm data generated from transfection or proteomic approaches.
- Use a selection of Signal-Seeker™ kits to build a PTM profile for your POI.
- Investigate the role of known protein 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 10 phosphotyrosine 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 1 bottle	Resuspend in 15 mls sterile water to give a 1X stock solution	4°C
BlastR™ Dilution Buffer	Part# BDB01-S 2 bottles	Resuspend bottle with 30 mls sterile water to give a 1X stock solution.	4°C
BlastR-2™ Wash Buffer	Part# BWB02-S 2 bottles	Resuspend bottle with 30 mls sterile water to give a 1X stock solution.	4°C
Tyrosine Phosphatase Inhibitor (Pre-activated sodium orthovanadate)	Part# PYI01 1 tube	1) Resuspend completely in 250 ul of water for a 200mM (200X) stock solution. 2) Aliquot each tube into 5 x 50 ul volumes (5 tubes total). This	-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
Phosphotyrosine Affinity Beads	Cat# APY03-Beads 1 tube	Resuspend each tube in 330 µl of 50% glycerol. Each IP assay uses 30 µl of bead slurry.	-20°C
Control beads (to assess non-specific binding)	Cat# CIG01-Beads 1 tube	Resuspend each tube in 330 µl of 50% glycerol. Each IP assay uses 30 µl of bead slurry.	-20°C
Anti-phosphotyrosine-HRP antibody	Cat# APY03-HRP-S (1 tube)	Resuspend in 25 µl of 50% glycerol	-20°C
Precision Red™ Advanced Protein Assay Reagent	Part #GL550 1 bottle (30 ml)	Not required	Room temp.
Bead Elution Buffer	Part# BEB01 1 tube (1.3 ml)	Not required	Room temp.
DMSO	Part# DMSO 1 tube (1.5 ml)	Not required	Room temp.
Chemiluminescent detection reagent A	Part # CLRA-S 1 bottle (4 ml)	Not required	Room temp.
Chemiluminescent detection reagent B	Part# CLRB-S 1 bottle (4 ml)	Not required	Room temp.
Spin columns Collection tubes	Part# TT89 2 packs of 10	Not required	Room temp.
BlastR™ filters	Cat# BLR02 10 filters	Not required	Room temp.

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

II: Kit Contents Cont'd

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

III: Assay Protocol

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 utilizing 150 cm² 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 <u>final</u> volume
1-2 mg	300 µl	To make 1.5 ml <u>final</u> volume
2-4 mg	600 µl	To make 3 ml <u>final</u> volume
4-6 mg	900 µl	To make 4.5 ml <u>final</u> 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	Sample 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 μ l 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 μ l 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 μ l of filtered lysate with 200 μ l 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 μ l 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 Phosphotyrosine Affinity Bead suspension several times to make sure that the beads are completely resuspended in the tube.
2. For each IP assay, aliquot 30 μ l 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 30 μ l of bead suspension per control reaction to determine non-specific binding. (Control IP tube).
5. Wash beads 2 times with 250 μ l 1X PBS-T to remove storage buffer.
6. Save a small amount of lysate (20 μ l) to run as a western input lysate control. Add 5 μ l 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 μ l 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 μ l 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

1. A primary antibody provided by the end user will be used for detection of the tyrosyl phosphorylated 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 chemiluminescence 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. 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 chemiluminescent detection reagents supplied in this kit is sufficient for 4 minigel 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 Tyrosyl Phosphorylated species in the IP

It is good practice to check the IPs for total phosphotyrosine species. This serves as a control assay to make sure that the IP reactions are efficiently enriching for tyrosyl phosphorylated proteins.

Re-probe Blot with anti-phosphotyrosine-HRP

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

NOTE: See Recommended Control Reaction section for instructions on how to make a strong positive control lysate with peroxide activated vanadate.

2. After a brief 10 minute wash in TBST at room temperature with shaking, incubate the membrane with a 1:6000 dilution of anti-phosphotyrosine-HRP antibody diluted in 3% milk (TBST) 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 phosphotyrosine protein species signal using x-ray film or CCD camera imaging.

IV: Troubleshooting

Observation	Possible cause	Remedy
No target protein tyrosyl phosphorylation signal detected	<p>There are several possible reasons for this result;</p> <ol style="list-style-type: none"> 1) The protein of interest is not tyrosyl phosphorylated 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 tyrosyl phosphorylation 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%). 4) Inefficient elution of captured PTM proteins from beads 	<ol style="list-style-type: none"> 1) Make sure that the affinity beads enriched for total phosphotyrosine species by using the anti-phosphotyrosine-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 tyrosine phosphatase inhibitor such as hydrogen peroxide activated orthovanadate (see recommended control reaction section) can be added to the cell culture 2-3 hours prior to harvesting. Inhibitor treatment allows the accumulation of phosphotyrosine 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 chemiluminescent detection reagent from the Signal-Seeker™ kit is being used. 4) It is very important to remove all of the final wash buffer prior to eluting the captured proteins from the beads. Diluting the elution buffer will greatly diminish its ability to elute captured proteins.
Band visible at >200kD in all IP samples	<p>The phosphotyrosine affinity beads are covalently linked to protein G-agarose to minimize leaching of antibody. Also a non-reducing bead elution buffer is used to minimize leaching of the antibody light chain. Occasionally a high molecular weight protein complex (protein G-linked antibody) can be detected in the western blot.</p>	<ol style="list-style-type: none"> 1) Run an Affinity Bead only sample that does not contain cell lysate. If the >200kD band is coming from the beads then it will be visible in this sample. If it does not interfere with your protein of interest it can be ignored. 2) Try using a rabbit primary antibody as these will not react with the leached mouse antibody.
Unmodified protein band detected in IP	<p>As there is often little or no obvious molecular weight difference between modified and unmodified proteins for phosphotyrosine modifications, it is very important to run the control bead IP to identify non-specific binding to beads.</p>	<ol style="list-style-type: none"> 1) Always run an IP Control Bead reaction to determine degree of non-specific binding. 2) Pre-clearing with IP control beads may reduce unmodified protein binding
No pellet or flocculent pellet after cell lysis and centrifugation	<p>If lysis volumes different from those given in the method are used it is possible to get a flocculent pellet after centrifugation of lysates.</p>	<p>Follow recommended lysis method see table 3.</p>

V: 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. Bunda S. et al. 2014. Src promotes GTPase activity of Ras via tyrosine 32 phosphorylation. *Proc. Natl. Acad. Sci. USA* **E3785-E3794**.

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: PKC α is degraded over a 4h period following PKC α 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 PKC α degradation.	1
Tau	Hyperphosphorylation 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 phosphorylation; a) Normal conditions promote p73 ubiquitination and degradation. b) Genotoxic stress promotes phosphorylation which inhibits ubiquitination and stabilizes p73.	4

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

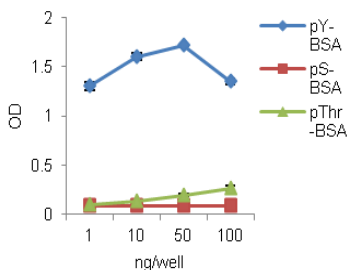
Technical Challenge	Signal-Seeker™ Solution
<p>The percentage modified vs unmodified protein under any physiological condition is typically very low (modified being only 1-2% of the unmodified protein amount). This is reflective of the fact that modifications often occur at the site of action in the 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.</p>	<p>Optimized sensitivity is a key feature of Signal-Seeker™ kits.</p> <ul style="list-style-type: none"> 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.
<p>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.</p>	<ul style="list-style-type: none"> a) The Signal-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.
<p>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.</p>	<p>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.</p>
<p>Buffer conditions are not compatible between different modifications.</p>	<p>Signal-Seeker™ 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.</p>
<p>PTM enrichment experiments are complex and exacting. They require high quality affinity matrices, optimized buffers and inhibitors and sensitive detection reagents.</p>	<p>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.</p>

Appendix III: Proprietary Phosphotyrosine Affinity Beads

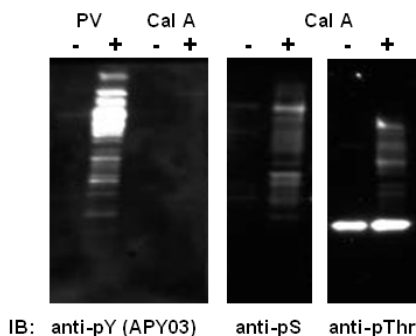
Signal Seeker™ Phosphotyrosine Affinity Beads

Phosphotyrosine Affinity Beads (Cat # APY03-Beads) are composed of a highly sensitive and specific anti-phosphotyrosine antibody (Cat # APY03) covalently linked to protein G-agarose. The APY03 antibody was developed by scientists at Cytoskeleton Inc. using a screen specifically designed to generate high affinity/specificity IP reagents. The data shown in figure 2 highlights the specificity of the beads. See Example data section for sensitivity data.

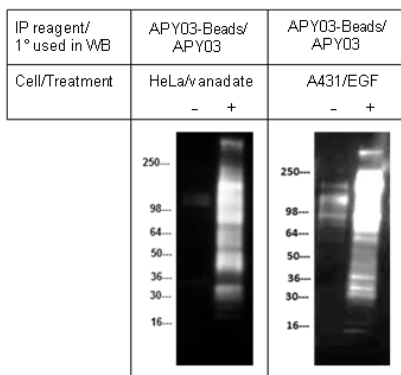
Figure 2: Specificity of Phosphotyrosine Beads for Tyrosyl Phosphorylated Proteins



Phosphopeptides phosphotyrosine (pY), phosphoserine (pS) and phosphothreonine (pThr) were conjugated to BSA, and increasing amounts of phosphopeptide-BSA were added serially to an ELISA plate. APY03 (1:5000) and goat anti-mouse (1:5000) were used to detect phosphopeptide-BSA in wells. OD data demonstrates specificity of APY03 to pY.



The western blot data shown in figure 2B demonstrates the specificity of APY03 for pY modified proteins. 20 µg of cell lysate from cells either left untreated or treated with 100mM of H₂O₂ activated pervanadate (PV), a tyrosine phosphatase inhibitor, for 10 minutes or 50nM of calyculin A, a serine/threonine phosphatase inhibitor, for 1 hour were resolved by SDS-PAGE and immunoblotted (IB) with the indicated antibodies.

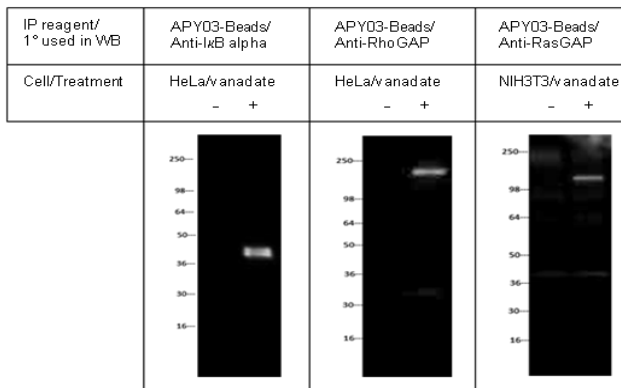


Western blot data shown in figure 2C demonstrates the specificity of APY03-Beads for tyrosyl phosphorylated proteins. HeLa cells were either treated or untreated with 100µM of H₂O₂ activated sodium orthovanadate for 30 minutes. A431 cells were serum starved for 24 hours before treatment with EGF (50ng/ml for 5 min). Lysates were immunoprecipitated using the Signal-Seeker™ Phosphotyrosine Enrichment kit.

Appendix IV: Example Data

The data shown below was generated using the Signal-Seeker™ Phosphotyrosine Enrichment kit. The target proteins detected are IκB alpha, Rho GAP, Ras GAP (Figure 3) and Rac1 respectively (Figure 4).

Figure 3: Detection of Endogenous Phosphotyrosine modified IκB alpha, RhoGAP and Ras GAP

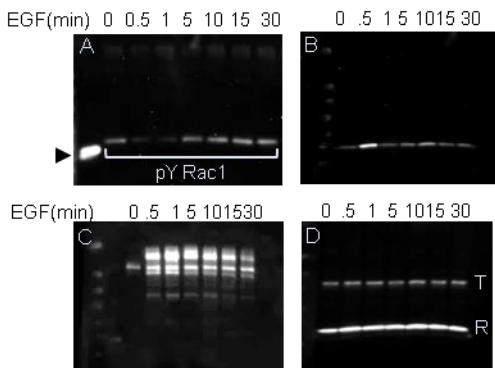


Hela cells were either treated or untreated with 100uM of H₂O₂ activated sodium othovanadate for 30 minutes. NIH3T3 cells were either treated or untreated with 100uM of H₂O₂ activated sodium othovanadate for 10 minutes. Cells were processed and analyzed using Signal-Seeker™ Phosphotyrosine Enrichment kit according to the kit protocol. Each lane represents results

from 1 mg of lysate. Primary antibodies, anti-IκB alpha (1:1000, BD Biosciences # 610690), anti-RhoGap (1:1000, Millipore # 05-378) and anti-RasGap (1:1000, BD Biosciences # 610040) and goat-anti-mouse secondary (1:20000, Jackson Labs # 115-035-068) were used in western blot analysis. Figure 3 shows that the assay can detect immunoprecipitated endogenous tyrosine phosphorylated IκB alpha (~38kDa) and RhoGAP (~190kDa) & RasGAP (~120kDa).

Figure 4: Time course Showing Rac1 Tyrosyl Phosphorylation and Activation

Signal-Seeker™ Phosphotyrosine Enrichment Kit was used to analyze the tyrosyl phosphorylation of Rac1 in



response to Epidermal Growth Factor (EGF) stimulation. HeLa cells were serum starved for 24h and either untreated (0) or treated with EGF (50ng/ml) for the indicated times. Figure 4A shows data generated using the Signal-Seeker™ kit and probing the blot with anti-Rac1 antibody. Tyrosyl Rac1 levels were consistently shown to dip over the first minute of EGF stimulation (repeated 5 times). Interestingly Rac1 activation, shown in Figure 4B increased as Rac1 tyrosyl phosphorylation decreased. This observation supports a model that has previously been suggested

for kinase regulation of Ras GTPase activity (5). Figure 4C shows the total phosphotyrosine signal during EGF stimulation and Figure 4D show that equal amounts of lysate were used for each IP and activation reaction (10 μl of each lysate was run and analyzed by western blotting using an anti-tubulin antibody (T) and an anti-Rac1 antibody @. Arrowhead points to total Rac1 signal from 3% of input lysate.

Appendix V: Test Plate

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 cm² 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 $OD_{600} \times 25 =$ 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 μ l). 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 (μ l)	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 μ l 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.

Appendix VI: Lysis and Dilution Buffer Prep

Lysis and Dilution Buffer Prep

Supplement the required volume of BlastR™ Lysis Buffer and BlastR™ Dilution Buffer with tyrosine phosphatase inhibitor (Part # PYI01) 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	985 µl
Tyrosine phosphatase Inhibitor (200x)	5 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
985 µl	1970 µl	4.925 ml	9.850 ml
5 µl	10 µl	25 µl	50 µl
10 µl	20 µl	50 µl	100 µl

Supplemented BlastR™ Dilution Buffer (4.0 ml)

BlastR™ Dilution Buffer	3,940 µl
Tyrosine phosphatase Inhibitor (200x)	20 µl
Protease Inhibitor cocktail (100x)	40 µl

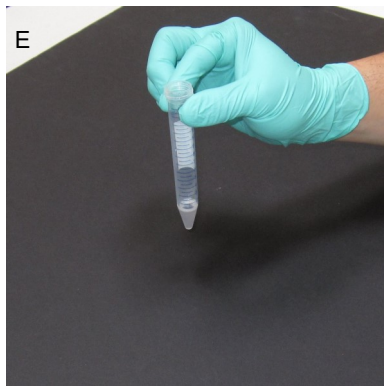
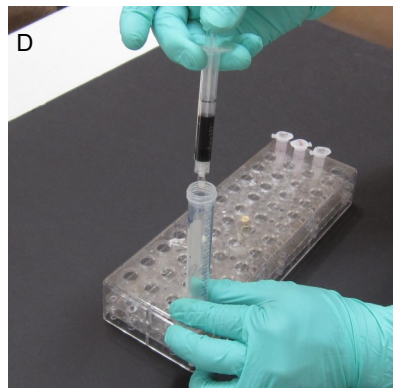
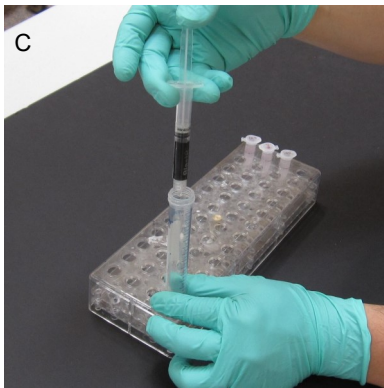
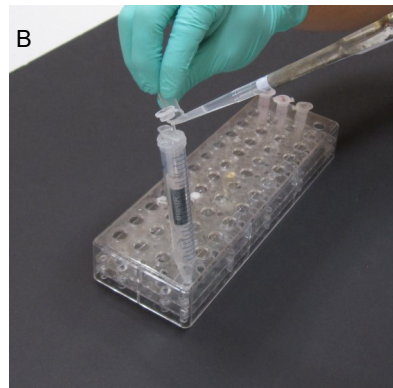
Table 8: Calculations for BlastR™ Dilution Buffer

4.0 ml	8.0 ml	20.0 ml	40.0 ml
3.960 ml	7.88 ml	19.7 ml	39.4 ml
20 µl	40 µl	100 µl	200 µl
40 µl	80 µl	200 µl	400 µl

Appendix VII: Utilization of BlastR™ Lysate Filter

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 a 15ml 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

Appendix VIII: Protein Quantitation Assay

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;

$$\text{sample reading OD}_{600} \times 5 = \text{protein concentration in mg/ml}$$

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

Appendix IX: Recommended Control Reactions

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

A) Phosphotyrosine IP Control Beads

Phosphotyrosine 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 IP Control Beads contained in this kit are composed of mouse IgG covalently bound to protein G agarose. Each kit contains sufficient beads to carry out 10 control IP reactions. Instructions for use are given in the assay protocol (STEP 3).

In some cases Control Beads will bind non-specifically to the unmodified protein of interest. The Signal-Seeker™ assay stringency is such that non-specific binding is rarely an issue. In cases where this is an issue 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 phosphotyrosine modified protein species

After probing the phosphotyrosine IP reactions with an antibody to the protein of interest it is recommended to re-probe the blot with an anti-phosphotyrosine antibody. This control reaction allows the end-user to confirm that the phosphotyrosine IP reaction has enriched for total tyrosyl phosphorylated species in the lysate. An HRP-conjugated anti-phosphotyrosine antibody is included in this kit (Cat# APY03-HRP-S) and instructions for use are given in the Western Blot protocol (STEP 4B).

It should be noted that endogenous levels of tyrosyl phosphorylated proteins can be extremely low in many cell lines and in the absence of a known stimulant of phosphotyrosine a strong positive signal will not be observed with the pan phosphotyrosine antibody. For a robust signal with total phosphotyrosine species we recommend treating a control plate as follows;

1. Grow cells (e.g. HeLa, NIH3T3 or A431) to about 80% confluent in a 150 cm² tissue culture dish.
2. Add 3 ul of hydrogen peroxide (30% W/V) to 22 ul of PBS in a microfuge tube. Add 25ul of pre-activated sodium orthovanadate (supplied in kit) to the mixture. The solution will turn yellow. Use immediately.
3. Add 15 ul of the hydrogen peroxide activated sodium orthovanadate to the dish. Mix well and incubate cells in tissue culture incubator. Treatment times for HeLa, NIH3T3 and A431 are 30, 10 and 15 minutes respectively.
4. Collect cell lysates as in STEP 2.
5. Use 20 ug of lysate for a western blot analysis or 100ug of lysate for IP as positive control.

VI: Appendix IX: Recommended Controls Cont'd

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.

D) Inhibitor Negative Control Sample

Dephosphorylation by tyrosine phosphatases occurs very rapidly in the absence of the phosphatase inhibitor that is included in this kit (Part # PY101). In cases where the phosphotyrosine signal is very weak or the primary detection antibody gives spurious bands in a western, the inclusion of an IP derived from a lysate that is processed in the absence of inhibitor (PY101) will often confirm the authenticity of the tyrosyl phosphorylated species.

VII: Changes Made from previous manual version

Changes made previous manual

- 1) We have included BlastR™ filters in this update. The filters quickly and easily reduce the viscosity of cell lysates by removal of genomic DNA while allowing proteins to pass straight through. BlastR™ filters allow a simpler and more reproducible lysate preparation.
- 2) We have emphasized the need to completely remove wash buffer in the final wash step. Leaving residual wash buffer on the beads will dilute the elution buffer and result in less PTM enriched proteins eluting from the beads.

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Protocol