V. 4.0

Signal-Seeker™ Phosphotyrosine Enrichment Kit

30 Assays

Cat. # BK160

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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 small selection of some recent publications in this area. The body of literature and recent publications such as those in Table 1 have generated a consensus 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

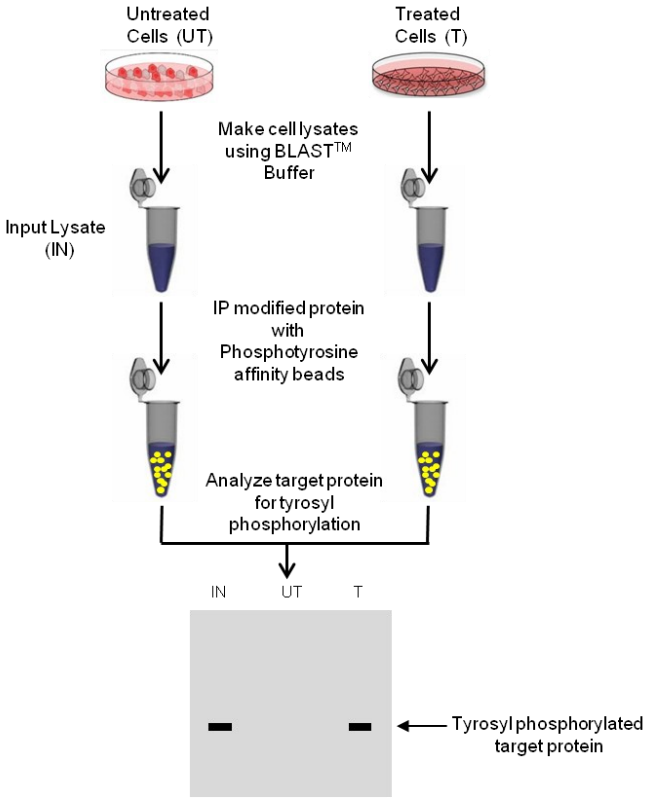
Protein	Co-dependent Modifications	Function	Ref.
Protein Kinase C (PKCα)	Phosphorylation SUMOylation Ubiquitination	**Observation:** PKCα is degraded over a 4h period following PKCa activation by the phorbol ester PMA. **Mechanism:** Mediated via a cascade of PTMs. A time-course for PMA treatment showed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

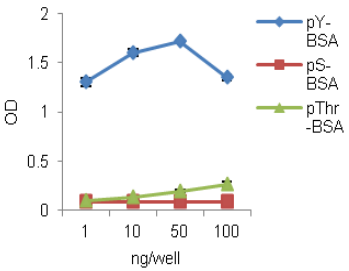
Technical Challenge	Signal-Seeker™ Solution
The percentage modified vs unmodified protein under any physiological condition is typically very low (modified being only 1-2% of the unmodified protein amount). This is reflective of the fact that modifications often occur at the site of action in the cell 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 feature of Signal-Seeker™ kits. a) Validation studies have demonstrated that Signal-Seekers™ can detect low level endogenous protein modifications (see Example Data section). b) High affinity IP beads and an optimized proprietary buffer system (BLAST™) 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) 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.
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 BLAST™ 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 for endogenous proteins 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 a simple and robust assay. The kits are accompanied by detailed and user-friendly instruction manuals and all of our products are supported by a knowledgeable technical support staff.

Signal Seeker™ Phosphotyrosine Affinity Beads

Phosphotyrosine Affinity Beads (Cat # APY03-Beads) are composed of a highly sensitive and specific anti-phosphotyrosine antibody (Cat # APY03) co-valently 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.

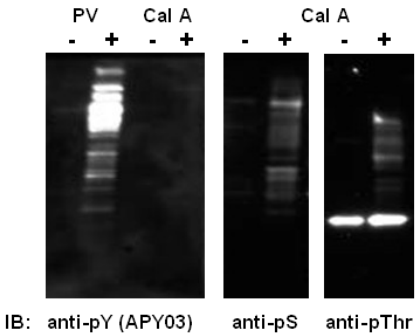
Figure 2: Specificity of Phosphotyrosine Beads for Tyrosyl Phosphorylated Proteins

A



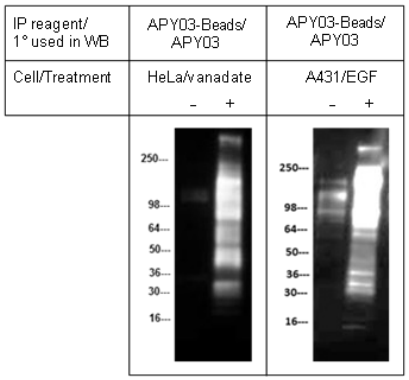
Phosphopetides phosphotyrosine (pY), phosphoserine (pS) and phosphothreonine (pThr) were conjugated to BSA, and increasing amounts of phosphopeptide-BSA were added serially to an ELISA plate. APY03 (1:5000) and goat anti-mouse (1:5000) were used to detect phosphopeptide-BSA in wells. OD data demonstrates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

B



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 H_2O_2 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.

C

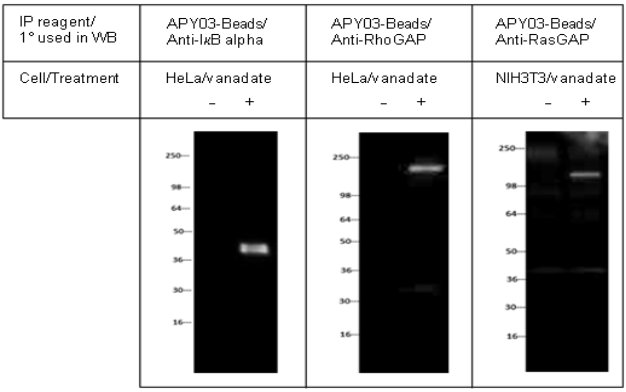


Western blot data shown in figure 2C demonstrates the specificity of APY03-Beads for tyrosyl phosphorylated proteins. HeLa cells were either treated or untreated with 100uM of H_2O_2 activated sodium othovanadate for 30 minutes. A431 cells were serum starved for 24 hours before treatment with EGF (50ng/ml for 5 min). Lysates were immunoprecipitated using the Signal-Seeker™ Phosphotyrosine Enrichment kit.

I: Introduction: Example Data

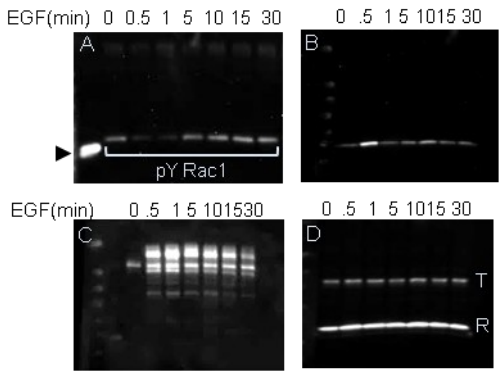
The data shown below was generated using the Signal-Seeker™ Phosphotyrosine Enrichment kit. The target proteins detected are IkB alpha, Rho GAP, Ras GAP (Figure 3) and Rac1 respectively (Figure 4).

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



Hela cells were either treated or untreated with 100uM of H_2O_2 activated sodium othovanadate for 30 minutes. NIH3T3 cells were either treated or untreated with 100uM of H_2O_2 activated sodium othovanadate for 10 minutes . Cells were processed and analysed using Signel-Seeker™ Phosphotyrosine Enrichment kit according to the kit protocol. Each lane represents results from 1 mg of lysate. Primary antibodies, anti-IkB 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 IkB 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 analyse 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 analysed 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.

II: Kit Contents

This kit contains enough reagents for 30 phosphotyrosine 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
BLAST™ Lysis Buffer	Part# BLST01	1 bottle	4°C desiccated
BLAST™ Dilution Buffer	Part# BDB01	1 bottle	4°C desiccated
BLAST™ Wash Buffer	Part# BWB01	1 bottle	4°C desiccated
Tyrosine Phosphatase Inhibitor (Pre-activated sodium orhtovanadate)	Part# PYI01	3 tubes	4°C desiccated
Protease Inhibitor Cocktail	Cat# PIC02	1 tube	4°C desiccated
Phosphotyrosine Affinity Beads	Cat# APY03-Beads	3 tubes	4°C desiccated
Control beads (mouse IgG covalently linked to agarose for analysis of non-specific binding)	Cat# CIG01-Beads	1 tube	4°C desiccated
Anti-phosphotyrosine-HRP antibody	Cat# APY03-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	1 tube (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

III: 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
BLAST™ Lysis Buffer	Resuspend in 15 mls sterile water to give a 1X stock solution	4°C
BLAST™ Dilution Buffer	Resuspend in 130 mls sterile water to give a 1X stock solution.	4°C
BLAST™ Wash Buffer	Resuspend in 130 mls sterile water to give a 1X stock solution.	4°C
Tyrosine Phosphatase Inhibitor (Pre-activated sodium orhtovanadate)	1) Resuspend each tube in 250 ul of water for a 200mM (**200X**) stock solution. Make sure powder is completely dissolved before use. 2) Aliquot each tube into 10 x 50 ul volumes (30 tubes total). 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
Phosphotyrosine Affinity Beads	Resuspend each tube in 330 µl of water. Each IP assay uses 30 µl of bead slurry.	4°C
Control Beads for IP	Resuspend each tube in 330 µl of water. Each IP assay uses 30 µl of bead slurry.	4°C
Anti-Phosphotyrosine -HRP antibody	Resuspnd in 25 µl of 50% glycerol	-20°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 Concentration

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 (approx. 14 cm diameter). Table 5 gives suggested lysis and dilution volumes per 150 cm^2 plate for given cell densities.

Table 5: BLAST™ Lysis/Dilution Buffer Chart

Cell Density	Recommended BLAST™ Lysis Buffer volume	Recommended BLAST™ Dilution Buffer volume
≥80%	300 µl	2-3 ml final volume, depending on desired protein concentration
30-79%	150 µl	1.5 ml final volume
< 30%	150 µl With sequential plate harvest to give a cumulative cell density of combined plates at >30%	1.5 ml final volume

2. Make up the following buffers as follows and place on ice. Volumes are per 150 cm^2 plate at high cell density. Supplemented buffers should be used within 1-2h. Unused buffer should be discarded.

   Supplemented BLAST™ Lysis Buffer (0.3 ml)

BLAST™ Lysis Buffer	294 µl
Tyrosine Phosphatase Inhibitor	3 µl
Protease Inhibitor cocktail	3 µl

   Supplemented BLAST™ Dilution Buffer (3.0 ml)

BLAST™ Dilution Buffer	2.94 ml
Tyrosine Phosphatase Inhibitor	30 µl
Protease Inhibitor cocktail	30 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 BLAST™

Lysis Buffer and make it less effective at lysing cells.

6. Lyse cells by adding 150-300 µl of supplemented BLAST™ 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 1.5ml tube (or similar) on ice. NOTE: at this point the total lysate volume should not exceed 2X the original lysate volume (e.g. 300 µl final volume for an original lysis volume of 150 µl).

8. Dilute the lysate to a total volume according to the BLAST™ Lysis/Dilution Buffer chart (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 BLAST™ Lysis Buffer and supplemented BLAST™ 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 150 µl of Lysis Buffer between plates (see Table 5).

STEP 2: 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 non-specific bead binding to the protein of interest. 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 4).

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 5B).

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 cm^2 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 3.
5. Use 20 ug of lysate for a western blot analysis or 100ug of lysate for IP as positive control.

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 BLAST™ Lysis Buffer and BLAST™ Dilution Buffer per 150 cm^2 tissue culture plate are given in Table 5.

3. Supplement the required volume of BLAST™ Lysis Buffer and BLAST™ Dilution buffer with Tyrosine Phosphatase inhibitor (10 µl per ml of buffer) and protease inhibitor cocktail (10 µl per ml of buffer). Remember you will require approximately 10X the volume of Dilution Buffer to Lysis Buffer. Recipes for 1 ml of Lysis Buffer and 10 ml of Dilution Buffer are given below. Final volumes required will need to be determined by the end-user.

   Supplemented BLAST™ Lysis Buffer (1.0 ml)

BLAST™ Lysis Buffer	980 µl
Tyrosine Phosphatase Inhibitor	10 µl
Protease Inhibitor cocktail	10 µl

   Supplemented BLAST™ Dilution Buffer (10 ml)

BLAST™ Dilution Buffer	9.8 ml
Tyrosine Phosphatase Inhibitor	100 µl
Protease Inhibitor cocktail	100 µl

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 BLAST™ Lysis Buffer and make it less effective at lysing cells.

10. Lyse cells by adding 150-300 µl of supplemented BLAST™ Lysis Buffer (see Table 2 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.

10. Transfer the lysate to a 1.5ml tube (or similar) on ice. NOTE: at this point the total lysate volume should not exceed 2X the original lysate volume (e.g. 300 µl final volume for an original lysis volume of 150 µl).
11. Dilute the lysate to a total volume according to the BLAST™ Lysis/Dilution Buffer chart (Table 5).
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 (14,000 rpm in a microcentrifuge), 4°C for 10 minutes. A visible 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 BLAST™ Lysis Buffer and supplemented BLAST™ 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 150 µl of Lysis Buffer between plates (see Table 5).
27. Equalize lysate protein concentrations using a 1:1 dilution of BLAST™ Lysis Buffer and BLAST™ 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 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 µl of bead suspension into a tube on ice.
3. Flick tube containing IP Control Bead suspension several times to make sure that the beads are completely resuspended in the tube.
4. Aliquot 30 µl of Control Bead suspension per control reaction to determine non-specific binding.
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 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. NOTE: volume should be 28-35 µl at this point. All sample volumes should be within ±10% of each other.

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 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 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 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 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 phosphotyrosine protein species signal using x-ray film or CCD camera imaging.

V: Troubleshooting

Observation	Possible cause	Remedy
No target protein tyrosyl phosphorylation signal detected	There are several possible reasons for this result; 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.	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) Protein tyrosyl phosphorylation can be very rapid and transient and can therefore be missed.	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 reatment allows the accumulation of phosphotyrosine species and increases chances of detecting low level, transient modifications.
	3) The amount of modified protein is typically only a small percent of the total protein (1-2%).	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 >200kD in all IP samples	The phosphotyrosine affinity beads are covalently linked to protein G-agarose to minimize leaching of antibody. Aslo 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.	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	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.	1) Always run an IP Control Bead reaction to determine degree of non-specific binding.
No pellet or flocculent pellet after cell lysis and centrifugatition	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.

VI: References

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

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