Cytoskeleton, Inc.
The Protein Experts

Manual

V.5.0

RhoA/Rac1/Cdc42 Activation Assay Combo Biochem Kit™

(30 Assays [10 Assays per small G-protein])

Cat. # BK030

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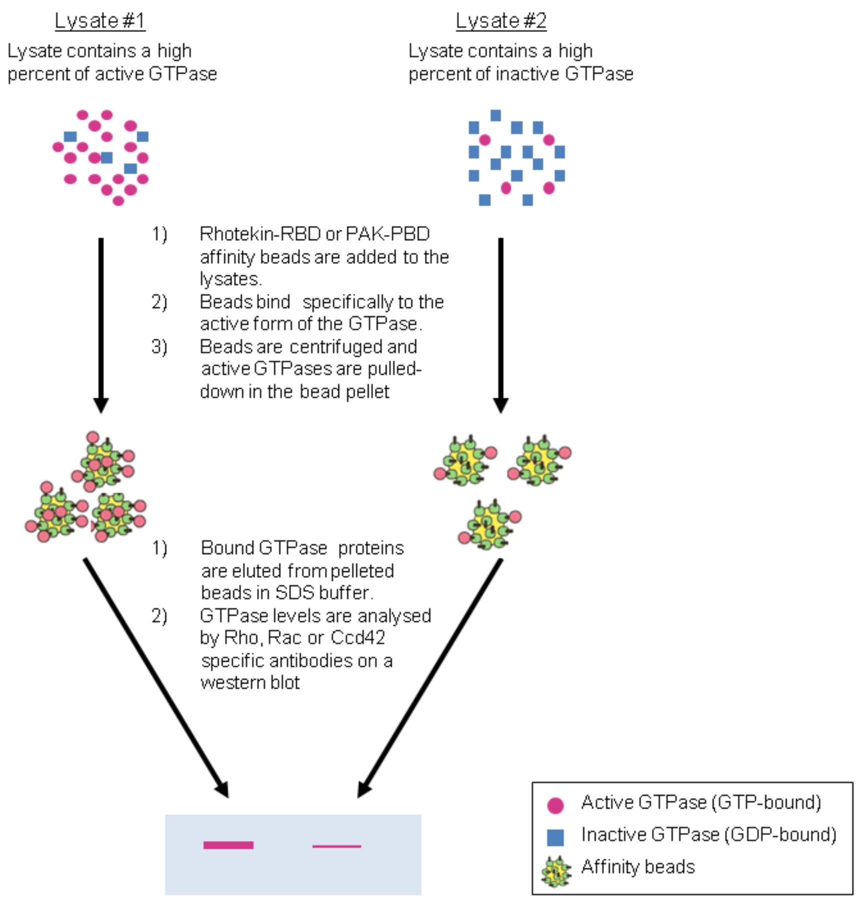
Manual Contents

Background– RhoA/Rac1/Cdc42 Activation Assays

The Rho family of small GTPases consists of at least 20 members, the most extensively characterized of which are the Rac1, RhoA and Cdc42 proteins (1-4). In common with all other small G-proteins, the Rho proteins act as molecular switches that transmit cellular signals through an array of effector proteins. This family mediates a diverse number of cellular responses, including cytoskeletal reorganization (1-4), regulation of transcription (5), DNA synthesis, membrane trafficking, and apoptosis (6-9). The Rho switch operates by alternating between an active, GTP-bound state and an inactive, GDP-bound state (10-12).

It is well documented that Rho family effector proteins will specifically recognize the GTP bound (active) form of the GTPase target (13). This has been exploited experimentally to develop powerful affinity purification Activation (Pull-down) Assays that monitor Rho family activation (14,15). The principle of the assay is shown schematically below (Figure 1). The remainder of this introduction provides a more detailed description of the three assays present in this combo kit (RhoA, Rac1 and Cdc42) .

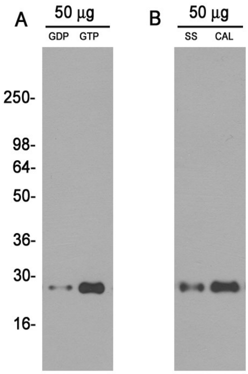
Figure 1: Schematic of RhoA/Rac1/Cdc42 Activation (Pull-Down) Assay



<u>RhoA Activation Assay (Utilizes Rhotekin-RBD Affinity beads (Part # RT02-S)</u>

This assay uses the Rho binding domain (also called the RBD) of the Rho effector protein rhotekin. The RBD protein motif has been shown to bind specifically to the GTP-bound form of Rho. The fact that the RBD region of rhotekin has a high affinity for GTP-Rho makes it an ideal tool for affinity purification of GTP-Rho from cell lysates. The rhotekin-RBD protein supplied in this kit contains amino acids 7-89 of rhotekin RBD expressed as GST fusion in *E.coli* bound to colored glutathione-sepharose beads. This allows one to “pull-down” GTP-Rho complexed with rhotekin-RBD beads. This assay provides a simple means of analyzing cellular Rho activities in a variety of systems. The amount of activated Rho is determined by a Western blot using a Rho specific antibody. A typical Rho pull-down assay using GTP and GDP loaded human platelet extracts or Swiss 3T3 cell extracts is shown in Figure 2.

Figure 2: RhoA Activation Assay



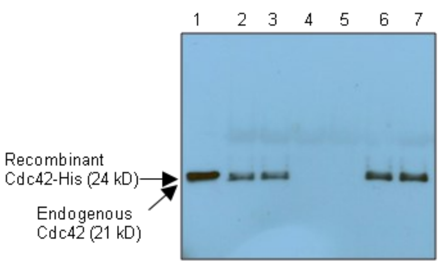
A. Extract (300 µg) from human platelet cells was loaded with GTPγS (GTP lane) or GDP (GDP lane) using the method described in Section VI: Control Reactions.

B. Extract (300 µg) from serum starved (SS) and subsequent calpeptin (CAL) treated Swiss 3T3 cells. All extracts were incubated with 50 µg of rhotekin-RBD beads and processed as described in Section VI: Pull-down Assay. All bead samples were resuspended in 10 µl of 2x sample buffer and run on a 12% SDS gel. Protein was transferred to PVDF, probed with a 1:500 dilution of anti-RhoA and processed for chemiluminescent detection as described in Section VI: STEP 4.

Rac1/Cdc42 Activation Assay (Utilizes PAK-PBD Affinity beads (Part # PAK02-S)

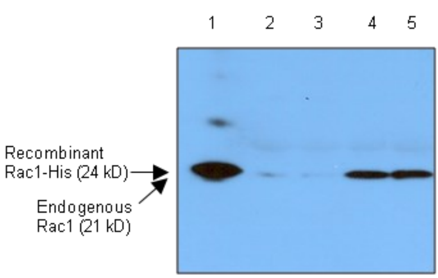
This assay uses the Cdc42/Rac Interactive Binding (CRIB) region (also called the p21 Binding Domain, PBD) of the Cdc42/Rac effector protein, p21 activated kinase I (PAK). The CRIB/PBD protein motif has been shown to bind specifically to the GTP-bound form of Rac and/or Cdc42 proteins (16). The fact that the PBD region of PAK has a high affinity for both GTP-Rac and GTP-Cdc42 and that PAK binding results in a significantly reduced intrinsic and catalytic rate of hydrolysis of both Rac and Cdc42 make it an ideal tool for affinity purification of GTP-Rac and GTP-Cdc42 from cell lysates (17). The PAK-PBD protein supplied in this kit contains amino acids 67-160, which includes the highly conserved CRIB region (aa 74-88) plus sequences required for the high affinity interaction with GTP-Rac and GTP-Cdc42 (17). The PAK-PBD is also in the form of a GST fusion protein which allows one to "pull-down" the PAK-PBD/GTP-Rac (or GTP-Cdc42) complex with glutathione affinity beads. The assay therefore provides a simple means of quantitating Rac/Cdc42 activation in cells. The amount of activated Rac1/Cdc42 is determined by a Western blot using a Rac1/Cdc42 specific antibody. Figures 3 and 4 show typical Cdc42 and Rac1 Activation Assay results from serum starved and EGF treated Swiss 3T3 cells. Serum starvation greatly reduces the basal amount of active Rac1/Cdc42 in cells while EGF is a potent activator of Rac1 and Cdc42.

Figure 3: Cdc42 Activation Assay



Swiss 3T3 cells were serum starved for 24h, after this some cells were treated with 100 ng/ml of EGF for 30 seconds (Lanes 2 & 3) or 2 minutes (Lanes 6 & 7), others were not treated and remained serum starved (Lanes 4 & 5). Cdc42 activation was measured using the Cdc42 Activation pull-down assay, 500 µg of lysate were assayed with 10 µg of PAK-PBD beads (Lanes 2-7). Lane 1 shows 20 ng of recombinant Cdc42-His protein run as a western blot standard. Note: the slight shadow signal running at approximately 36 kD in the pull-down lanes is signal from the PAK bead protein.

Figure 4: Rac1 Activation Assay



Swiss 3T3 cells were serum starved for 24h, after this some cells were treated with 10 ng/ml of EGF for 2 min. (Lanes 4 & 5), others were not treated and remained serum starved (Lanes 2 & 3). Rac1 activation was measured using the Rac1 Activation pull-down assay, 500 µg of lysate were assayed with 10 µg of PAK-PBD beads (Lanes 2-5). Lane 1 shows 20 ng of recombinant Rac1-His protein run as a western blot standard.

II: Purchaser Notification

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.

III: Kit Contents

This kit contains enough reagents for approximately 10 pull-down assays each for RhoA, Rac1 and Cdc42.

Table 1A: Rho A Specific Reagents

Reagents	Cat. # or Part # *	Quantity	Storage
Rhotekin RBD beads	Part # RT02-S	1 tube, lyophilized; 0.5 mg of protein per tube bound to colored sepharose beads	Desiccated 4°C
Anti-RhoA monoclonal antibody	Cat # ARH04	1 tube, lyophilized	Desiccated 4°C
His-RhoA control protein	Part # RHWT	1 tube, lyophilized; 10 µg protein (~30 kDa) as a Western Blot standard.	Desiccated 4°C

Table 1B: Rac1/Cdc42 Specific Reagents

Reagents	Cat. # or Part # *	Quantity	Storage
PAK-PBD beads	Part # PAK02-S	1 tube, lyophilized; 0.2 mg of protein per tube bound to colored sepharose beads	Desiccated 4°C
Anti-Rac1 monoclonal antibody	Cat # ARC03	1 tube, lyophilized	Desiccated 4°C
His-Rac1 control protein	Part # RCWT	1 tube, lyophilized; 10 µg protein (~25 kDa) as a Western Blot standard.	Desiccated 4°C
Anti-Cdc42 monoclonal antibody	Cat # ACD03	1 tube, lyophilized	Desiccated 4°C
His-Cdc42 control protein	Part # CDWT	1 tube, lyophilized; 10 µg protein (~25 kDa) as a Western Blot standard.	Desiccated 4°C

Kit Content Tables continued on next page

III: Kit Contents (Continued)

Table 1C: General Kit Reagents

Reagents	Cat. # or Part #*	Quantity	Storage
Cell Lysis Buffer	Part # CLB01	1 bottle, lyophilized; 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, and 2% Igepal when reconstituted	Desiccated 4°C
Wash Buffer	Part # WB01-S	1 bottle, lyophilized; 25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl when reconstituted	Desiccated 4°C
Loading Buffer	Part # LB01	1 tube, 1 ml; 150 mM EDTA solution	4°C
STOP Buffer	Part # STP01	1 tube, 1 ml; 600 mM $MgCl_2$ solution	4°C
GTPγS stock: (non -hydrolysable GTP analog)	Cat # BS01	1 tube, lyophilized; 20 mM solution when recon- stituted	Desiccated 4°C
GDP stock	Part # GDP01	1 tube, lyophilized; 100 mM solution when recon- stituted	Desiccated 4°C
Protease Inhibitor Cocktail	Cat. # PIC02	1 tube, lyophilized; 100X solution: 62 µg/ml Leupeptin, 62 µg/ml Pepstatin A, 14 mg/ ml Benzamidine and 12 mg/ ml tosyl arginine methyl ester when reconstituted	Desiccated 4°C
DMSO	Part # DMSO	1 tube, 1.5ml. Solvent for protease inhibitor cocktail	4° (will freeze at 4°C)

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

III: Kit Contents (Continued)

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

- Cell lysate (see Section V: B-D and Section VI: Step 2)
- 2X Laemmli sample buffer (125 mM Tris pH 6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol blue, 5% beta-mercaptoethanol)
- Polyacrylamide gels (12% or 4-20% gradient gels)
- SDS-PAGE buffers
- Western blot buffers (see Section VI: Step 4)
- Protein transfer membrane (PVDF or Nitrocellulose)
- Secondary antibody (e.g., Goat anti-mouse HRP conjugated IgG, Jackson Labs. Cat# 115-035-068)
- Chemiluminescence based detection system (e.g., SuperSignal West Dura Extended Duration Substrate; ThermoFisher)
- Cell scrapers
- Liquid nitrogen for snap freezing cell lysates

IV: Reconstitution and Storage of Components

Many of the components of this kit have been provided in lyophilized form. Prior to beginning the assay you will need to reconstitute several components as detailed in Table 2. When properly stored and reconstituted, components are guaranteed stable for 6 months.

Table 2: Component Storage and Reconstitution

Kit Component	Reconstitution	Storage Conditions
Rhotekin-RBD beads	Reconstitute in 300 µl distilled water. Aliquot into 10 x 30 µl volumes (30 µl of beads = 50 µg of protein, under these conditions 300 µl is sufficient for 10 assays). **Snap freeze in liquid nitrogen**	Store at –70°C
Anti-RhoA monoclonal antibody	Resuspend in 200 µl of PBS. Use at 1:500 dilution	Store at 4°C
His-RhoA control protein	Reconstitute in 30 µl of distilled water. Aliquot into 10 x 3 µl sizes and snap freeze in liquid nitrogen.	Store at –70°C
PAK-PBD beads	Reconstitute in 200 µl distilled water. Aliquot into 20 x 10 µl volumes (10 µl of beads = 10 µg of protein, under these conditions 200 µl is sufficient for 20 assays). **Snap freeze in liquid nitrogen**	Store at –70°C
Anti-Rac1 monoclonal antibody	Resuspend in 100 µl of PBS. Use at 1:500 dilution	Store at 4°C
His-Rac1 control protein	Reconstitute in 30 µl of distilled water. Aliquot into 10 x 3 µl sizes and snap freeze in liquid nitrogen.	Store at –70°C
Anti-Cdc42 monoclonal antibody	Resuspend in 200 µl of PBS. Use at 1:250 dilution	Store at 4°C
His-Cdc42 control protein	Reconstitute in 30 µl of distilled water. Aliquot into 10 x 3 µl sizes and snap freeze in liquid nitrogen	Store at –70°C
Protease Inhibitor Cocktail	Reconstitute in 1 ml of dimethyl sulfoxide (DMSO) for 100x stock.	Store at –20°C.
Cell Lysis Buffer	Reconstitute in 100 ml of sterile distilled water. This solution may take 5-10 min to resuspend. Use a 10 ml pipette to thoroughly resuspend the buffer.	Store at 4°C
Wash Buffer	Reconstitute in 30 ml of sterile distilled water.	Store at 4°C
Loading Buffer	No reconstitution necessary.	Store at 4°C
STOP Buffer	No reconstitution necessary.	Store at 4°C
GTPγS stock (non-hydrolysable GTP analog)	Reconstitute in 50 µl of sterile distilled water. Aliquot into 5 x 10 µl volumes, snap freeze in liquid nitrogen.	Store at –70°C
GDP Stock	Reconstitute in 50 µl of sterile distilled water. Aliquot into 5 x 10 µl volumes, snap freeze in liquid nitrogen.	Store at –70°C

V: Important Technical Notes

A) **<u>Notes on Updated Version 4.1</u>**

The following update should be noted:

1. The RhoA Antibody has been changed from part #ARH03 to ARH04. ARH04 is a monoclonal anti-RhoA specific antibody. It has the same specificity as Cat# ARH03 and was found to give a more robust signal than ARH03.

B) **<u>Growth and Treatment of Cell Lines</u>**

The health and responsiveness of your cell line is the single most important parameter for the success and reproducibility of Rho family activation assays. Time should be taken to read this section and to carefully maintain cell lines in accordance with the guidelines given below.

Adherent fibroblast cells such as 3T3 cells should be ready at 30% confluency or for non-adherent cells, at approximately 3 x 10^5 cells per ml. Briefly, 3T3 cells are seeded at 5 x 10^4 cells per ml and grown for 3-5 days. Serum starvation (see below) or other treatment should be performed when cells are approximately 30% confluent. It has been found that cells cultured for several days (3-5 days) prior to treatment are significantly more responsive than cells that have been cultured for a shorter period of time. Other cell types may require a different optimal level of confluency to show maximum responsiveness to RhoA/Rac1/Cdc42 activation. Optimal confluency prior to serum starvation and induction should be determined for any given cell line (also see Appendix 2 for cell line specific references).

When possible, the untreated samples should have cellular levels of RhoA/Rac1/Cdc42 activity in a “controlled state”. For example, when looking for RhoA/Rac1/Cdc42 activation, the “controlled state” cells could be serum starved. Serum starvation will inactivate cellular Rho family proteins and lead to a much greater response to a given activator. A detailed method for serum starvation is given in Appendix 1.

Cells should also be checked for their responsiveness (“responsive state”) to a known stimulus. A list of known RhoA/Rac1/Cdc42 stimuli are given in Appendix 2. In many cases, poor culturing technique can result in essentially non-responsive cells. An example of poor culturing technique includes the sub-culture of cells that have previously been allowed to become overgrown. For example, Swiss 3T3 cells grown to >70% confluency should not be used for Rho family activation studies.

To confirm the “controlled state” and “responsive state” of your cells, it is a good idea to include a small coverslip in your experimental tissue culture vessels and analyze the “controlled state” cells versus the “responsive state” cells by rhodamine phalloidin staining of actin filaments. A detailed method for actin staining is given in Appendix 1.

If you are having difficulty determining a “controlled state” for your experiment then contact technical assistance at 303-322-2254 or e-mail tservice@cytoskeleton.com.

C) **Timing and Intensity of Rho Family Activation**

Upon stimulation, RhoA, Rac1 and Cdc42 proteins are generally activated very rapidly and transiently. Maximal activation ranges from 30 s to 30 min and declines thereafter to basal levels. Examples of known Rho family activators are given in Appendix 2. For potent activators, the intensity of maximal Rho family activation over "control state" (serum starved) cells is generally in the order of 2-5 fold (see Appendix 2). However, using a single time point, you are more likely to miss this maximum. It is therefore critical to take timed samples for at least the first experiment with an unknown activating entity. Recommended time points are 0, 1, 3, 6, 12 and 30 minutes (a time course is also recommended for Rho family inactivation studies).

In practical terms the timed experiment must be performed sequentially. This allows rapid processing of each single time point. Once one time point lysate is collected, it should be snap frozen in "experiment sized" aliquots immediately and kept at –70°C. The Activation Assay uses approximately 300-800 µg of total protein per assay; this translates to 600-1600 µl of a 0.5 mg/ml cell lysate. We recommend duplicate samples per time-point or condition, therefore 1.2– 3.2 ml aliquots are recommended for snap freezing.

D) **Rapid processing of cells**

GTP bound (active) Rho family proteins are labile entities and the bound GTP is susceptible to hydrolysis by GAPs during and after cell lysis, resulting in inactivation of the small G-protein. Rapid processing at 4°C is essential for accurate and reproducible results. The following guidelines are useful for rapid washing of cells.

Washing

a. Retrieve culture dish from incubator, immediately aspirate out all of the media and place firmly on ice.

b. Immediately rinse cells with an appropriate volume of ice cold PBS to remove serum proteins (see Table 3 for recommended wash volumes).*

c. Aspirate off all residual PBS buffer. This is essential so that the Lysis Buffer is not diluted. Correct aspiration requires that the culture dish is placed at a steep angle on ice for 1 min to allow excess PBS to collect in the vessel for complete removal.

*NOTE: In 3T3 cells, it has been found that omitting the wash step results in a more reproducible Cdc42 activation. However, if the final growth media contains proteins then the wash step can not be omitted as the protein content of the media will interfere with cell lysate protein quantitations.

Cell Lysis

To avoid making too dilute or too concentrated lysate samples (<0.25 or >2.0 mg/ml), it is recommended to adjust the amount of Cell Lysis Buffer depending on your cell type and plate type. Table 3 gives guidelines for suitable lysis volumes for 3T3 cells which tend to give low protein yields. The exact lysis volumes for any given cell line will have to be determined empirically. NOTE: Cell Lysis Buffer should contain 1X Protease Inhibitor Cocktail.

Table 3: Recommended Wash and Lysis Volumes for 3T3 Cell Culture

Culture Vessel	**Vessel surface area (cm^2)**	**Volume of PBS wash (ml)**	**Volume of Lysis Buffer (µl)**
100 mm dish	56	10.0	250
150 mm dish	148	15.0	700
T-75 Flask	75	10.0	500
T-150 Flask	150	15.0	700

The time period between cell lysis and addition of lysates to the affinity beads is critically important. Take the following precautions:

1. Work quickly.

2. Keep solutions and lysates <u>embedded</u> in ice so that the temperature is below 4°C. This helps to minimize changes in signal over time. The Assay Protocol (Section VI) gives very specific instructions regarding temperature and must be strictly adhered to for successful results.

3. We strongly recommend that cell lysates be immediately frozen after harvest and clarification. A sample of at least 20 µl should be kept on ice for protein concentration measurement. A 20-50 µg aliquot of each sample should also be kept for Western blot quantitation of total RhoA, Rac1 or Cdc42. Cytoskeleton also provides an ELISA quantitation for total RhoA (see Appendix 4). The lysates **<u>must</u>** be snap frozen in liquid nitrogen and stored at -70°C. Lysates can be stored at -70° C for several months.

4. Thawing of cell lysates prior to use in the pull-down assay should be in a room temperature water bath, followed by rapid transfer to ice and immediate use in the assay.

E) **<u>Protein Concentration Equivalence</u>**

Equal protein concentration in all samples is a prerequisite for accurate comparison between samples in Rho family activation assays. Cell extracts should be equalized with <u>ice cold</u> Cell Lysis Buffer to give identical protein concentrations. For example, cell lysates of protein concentrations ranging from 0.5–1.3 mg/ml would all need to be diluted to 0.5 mg/ml. It is not necessary to equalize protein concentrations if the variation between them is less than 10%.

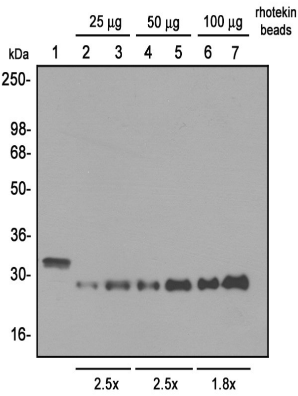
To quantitate the amount of total small G-protein, we recommend including samples of total lysate from experimental samples in a Western blot. Samples of 20-50 µg total cell lysate per sample should be sufficient to detect total RhoA, Rac1 or Cdc42. Alternatively, total RhoA can be quantitated by a RhoA ELISA (see Appendix 4).

F) **Assay Linearity**

There are several factors to consider when performing the Rho family activation assays:

1) **Bead Titration:** The PAK-PBD and Rhotekin-RBD affinity beads will bind to the inactive (GDP-bound) small G-protein with a much lower affinity than the active (GTP-bound) protein. If too many affinity beads are added to the pull-down assay there will be significant binding to the inactive (GDP-bound) small G-protein. The result of this will be an underestimation of small G-protein activation. For this reason we highly recommend performing a bead titration to determine optimal conditions for any given activation or inactivation assay. Once optimal conditions have been established, bead titrations should no longer be necessary. We recommend 25, 50 and 100 µg bead titrations for Rhotekin-RBD (RhoA activation, see Figure 4). We recommend 10 and 20 µg bead titrations for PAK-PBD (Rac1 and Cdc42 activation).

Figure 4: Rhotekin-RBD Bead Titration on Serum Starved and Calpeptin Treated Swiss 3T3 Cell Extracts.



Frozen cell lysates of serum starved (samples 2, 4 and 6) and calpeptin treated (samples 3, 5 and 7) cells were prepared according to the method described in Appendix 1. Lysates (300 µg ach at 0.5 mg/ml) were incubated with increasing amounts of rhotekin-RBD beads (25, 50 or 100 µg) and processed as described in Section VI:STEP 3: Pull-down Assay. All bead samples were resuspended in 12 µl of 2x Laemmli sample buffer and run on a 12% SDS gel along with 20 ng (lane 1) of His-RhoA control protein. Protein was transferred to PVDF, probed with a 1:500 dilution of anti-RhoA and processed for chemiluminescent detection.

Note, in Figure 4, how increasing amounts of rhotekin-beads result in more “non-specific” RhoA signal in the serum starved samples (compare lanes 4 and 6). RhoA activation estimates of 2.5 fold are observed using 25 and 50 µg of rhotekin-beads, whereas a RhoA activation estimate of 1.8 fold is observed with the highest amount of beads (100 µg).

It is highly recommended that each user titrate the affinity beads for their particular experiment as cell lysate concentration, G-protein activation efficiency, bead binding and processing times can be variable and must be optimized for reproducible results.

2) Strictly Maintain Experimental Conditions: Once assay conditions are established one should strictly maintain experimental conditions. For example, lysate concentrations should be consistent between experiments. Thus, if 50 µg of beads are used to assay 400 µg of lysate at 0.5 mg/ml protein concentration, it is recommended to keep subsequent assays at 0.5 mg/ml lysate rather than using half the volume of a 1 mg/ml lysate to give 400 µg total protein. As a further example, the growth and treatment of cell lines should be consistent between experiments; this point can not be over-emphasized and is discussed in detail in Section V: B.

V: Important Technical Notes (Continued)

3) **Densitometric Quantitation:** The linear range of X-ray film is very narrow. Multiple exposures of the western blot may be required to analyze data in the linear range of the film. As a general guideline, protein bands that appear grey rather than black will be within the linear range of the film.

STEP 1: Control Reactions

The correct control reactions are key components of the Rho family Activation Assay. The following control assays should be performed as an integral part of each experiment:

1. **Total Small G-Protein:**

   Total RhoA, Rac1 or Cdc42 present in each sample should be determined by Western quantitation or ELISA (for RhoA; see Appendix 4). Usually 20 – 50 µg of cell lysate will result in a good signal. Normalization of active GTPase against total GTPase is an important parameter in understanding the mechanisms underlying GTPase activity (see Appendix 4, and information at www.cytoskeleton.com).

2. **Positive Cellular Protein Control**:

   Total cell lysate (300 – 800 µg) should be loaded with GTPγS as a positive control for the pull-down assay. The following reaction details how to load endogenous RhoA, Rac1 and Cdc42 with the non-hydrolysable GTP analog (GTPγS), this is an excellent substrate for rhotekin-RBD and PAK-PBD beads and should result in a strong positive signal in a pull-down assay.

   a. Perform GTP loading on 300 – 800 µg of cell lysate by adding 1/15th volume of Loading Buffer (70 µl Loading Buffer per ml of lysate).
   b. Immediately add 1/100th volume of GTPγS (10 µl GTPγS per 990 µl of lysate) to give a 200 µM final GTPγS concentration. Under these conditions, 5 - 10% of the small G-protein will be activated and result in a strong affinity bead pull-down assay signal.
   c. Incubate the control sample at room temperature for 15 min with gentle rotation.
   d. Stop the reaction by transferring the tube to 4°C and adding 1/10th volume of STOP Buffer (100 µl STOP Buffer per 900 µl of lysate) .
   e. Use this sample immediately in a pull-down assay as detailed in STEP 3.

3. **Negative Cellular Protein Control:**

   This reaction should be performed in an identical manner to the Positive Control reaction except that 1/100th volume of GDP (1 mM final concentration) should be added to the reaction in place of the GTPγS. Loading endogenous small G-proteins with GDP will inactivate them and this will bind very poorly to affinity beads.

4. **His-tagged small G-protein Controls:**

   The kit supplies 10 µg of His-tagged RhoA, Rac1 and Cdc42 control protein; these will be reconstituted to a 0.33 mg/ml stock solution and stored at -70°C (as 10 x 3 µl aliquots). Storage of the protein at lower concentrations than 0.33 mg/ml or freeze/ thaw cycles will result in denaturation, precipitation of the protein and incorrect quantitations or no signal in the western blot. The Rho family proteins have a molecular weight of between 20-25 kDa; the His-tagged control proteins have molecular weights between 23-30 kDa. We recommend that 20 ng of His-tagged control protein be run on the gel as a positive control and as a quantitation estimate for endogenous small G-protein (see STEP 4).

STEP 2: Lysate Collection

We strongly recommend that you snap freeze your cell lysates in liquid nitrogen right after you harvest and clarify. This is especially necessary if you have many samples. It is recommended to freeze lysates in 1-3 ml aliquots and to save a small amount of each lysate (approximately 20 – 30 µl) for protein quantitation. Details of lysates processing are given below:

Cells Grown in Tissue Culture Vessels as Monolayers

1. Grow cells in appropriate culture conditions. It is important to keep cells in a "controlled state" prior to activation. See Section V (B): Important Technical Notes.
2. Treat cells with a small G-protein activator (or inactivator) as your experiment requires.
3. After treatment, place culture vessel on ice, aspirate media, wash with ice cold PBS. See Table 3, Section V: D for recommended volumes. NOTE: In some cases, if there are no proteins in the growth media, the wash step can be omitted for Cdc42 activation. (see Section V [B]).
4. Aspirate off PBS. Tilt plates on ice for an additional 1 min to remove all remnants of PBS. Residual PBS will adversely affect the assay.
5. Lyse cells in an appropriate volume of ice-cold Cell Lysis Buffer (Lysis Buffer should be supplemented with 1X Protease Inhibitor Cocktail). See Table 3, Section V: D for recommended volumes.
6. Harvest cell lysates with a cell scraper. It is useful to incline the culture plate for this method because the Lysis Buffer is spread thinly on the surface.
7. Transfer lysates into the pre-labeled sample tubes on ice.
8. Immediately clarify by centrifugation at 10,000 x g, 4°C for 1 min.
9. At this point each lysate volume should not exceed 130% of the original Cell Lysis Buffer volume.
10. Save at least 20 µl of lysate for protein quantitation and 20-50 µg of lysate for quantitation of the total specific small G-protein.
11. Aliquot and snap freeze the remaining cell lysates in liquid nitrogen. Store at -70°C for future use. It is recommended to aliquot into 1-3 ml of lysate per tube (This should be sufficient for duplicate assays of 300-800 µg per assay).
12. Measure lysate protein concentrations. We recommend using Precision Red Advanced Protein Assay (Cat. # ADV02) for quantitations (see Appendix 3):
    - Add 20 µl of each lysate or Lysis Buffer into disposable 1 ml cuvettes.
    - Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Cat # ADV02) to each cuvette.
    - Incubate for 1 min at room temperature.
    - Blank spectrophotometer with the Cell Lysis Buffer at 600 nm.
    - Read absorbance of lysates samples.
    - Multiply the absorbance by 5 to obtain the protein concentration in mg/ml.

13. Calculate how to equalize the cell extracts with ice cold Lysis Buffer to give identical protein concentrations. It is essential to have equal protein concentration in each sample for a successful assay. It is also important that the equalized protein concentration is not higher than 2.0 mg/ml or below 0.25 mg/ml. It is not necessary to equalize protein concentration if the sample variation is less than 10%.

    The volume of cold cell lysis buffer to be added to the more concentrated samples can be calculated as follows:

    $$\frac{A - B}{B} \times \text{(volume of A)} = \_\_\_\_\_\_\_\_\_\_ \ \mu l$$

    Where A is the higher concentration lysates (mg/ml) and B is the concentration of the most dilute sample (mg/ml)

NOTE: You can dilute the lysates to a given concentration (e.g. 0.5 mg/ml) prior to snap freezing aliquots. This makes subsequent pull-down assays simpler. Be aware of the length of time cell lysates stay on ice (should not exceed 10 min), since Rho/Rac/Cdc42 GTP hydrolysis will occur.

STEP 3: Pull-down Assay

1. If using freshly prepared cell lysates, use as soon as possible after lysis and protein equalization and always maintain samples at 4°C. If using frozen lysates (recommended), thaw in a room temperature water bath and remove immediately to ice upon thawing. Use immediately.

2. Add equivalent protein amounts of lysate (300 – 800 µg total cell protein) to a pre-determined amount of rhotekin-RBD (for RhoA activation assay) or PAK-PBD beads (for Rac1 and Cdc42 activation assays) from your bead titration test (see Section V.F.1).

NOTE: In general, we recommend using 50 µg (30 µl) of rhotekin-RBD beads and 10 µg (10 µl) of PAK-PBD beads per assay. Under these conditions the 0.5 mg of rhotekin-RBD beads supplied in the kit is sufficient for 10 assays and the 0.2 mg of PAK-PBD is sufficient for 20 assays (sufficient for 10 Rac1 and 10 Cdc42 assays).

3. Incubate at 4°C on a rotator or rocker for 1 h.

4. Pellet the beads by centrifugation at 3-5,000 x g at 4°C for 1 min.

5. Very carefully remove 90% of the supernatant. Do not disturb the bead pellet. If you do disturb the pellet simply re-centrifuge the sample as in step 4.

6. Wash the beads once with 500 µl each of Wash Buffer. **NOTE**: Add the buffer to the bead pellet in a manner that completely resuspends the beads. **DO NOT invert the tube as the beads will disperse over the surface of the tube and protein will be lost. This step should take less than 1 min to perform.**

7. Pellet the beads by centrifugation at 3-5,000 x g at 4°C for 3 min.

8. Very carefully remove the supernatant. Do not disturb the bead pellet. If you do disturb the pellet simply re-centrifuge the sample as in step 7.

9. Add 10-20 µl of 2x Laemmli sample buffer to each tube and thoroughly resuspend the beads. Boil the bead samples for 2 min.

10. The samples are now ready to be analyzed by SDS-PAGE and Western blot analysis (see STEP 4).

NOTE: The samples can be centrifuged (14K rpm (16K x g), 2 minutes, room temperature) to pellet the beads; in this case only the supernatant will be loaded onto the gel. Alternatively, the whole sample including the beads can be loaded onto the gel. It is recommended that the necessary control samples be run on each gel.

STEP 4: Western Blot Protocol

1. Run the test protein samples and controls on a 4-20% or 12% SDS gel until the dye front reaches the bottom of the gel.
2. We recommend running a lane containing 20 ng of His-tagged control protein (RhoA, Rac1 or Cdc42, depending upon the specific antibody to be used in western analysis) as a positive control. To do this, the protein should be diluted as follows:
    a) Thaw one of the 3 µl aliquots of His-tagged control protein (see Table 2).
    b) Dilute to 4 ng/µl by adding 247 µl of Cell Lysis Buffer.
    c) Dilute to 2 ng/µl by adding 250 µl of 2X Laemmli sample buffer (125 mM Tris pH 6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol blue, 5% beta-mercaptoethanol).
    d) Load 10 µl (20 ng).
    e) Discard any unused control protein as it will "crash out" during storage at 4°C or frozen.
3. Equilibrate the gel in Western blot buffer (See recipe below) for 15 min at room temperature prior to electro-blotting.
4. Transfer the protein to a PVDF membrane for 45 minutes at 75V.
5. Wash the membrane once with TBS (10 mM Tris-HCl pH 8.0, 150 mM NaCl).
6. Allow the membrane to air dry for 20-30 minutes.
7. Transfer the membrane to TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) at room temperature for 15 minutes to rehydrate the membrane.
8. Block the membrane surface with 5% nonfat-dry milk in TBST for 30 min at room temperature with constant agitation.
9. Incubate the membrane with a **1:500** dilution of anti-RhoA, **1:500** anti-Rac1 or **1:250** anti-Cdc42 antibody diluted in TBST (no blocking agent for RhoA or Rac1 antibody. Include 0.1% nonfat dry milk with the Cdc42 antibody) for 2-3 h at room temperature or overnight at 4°C with constant agitation.
10. Rinse the membrane in 50 ml TBST for 1 min.
11. Incubate the membrane with an appropriate dilution (eg. 1:20,000) of anti-mouse secondary antibody (eg. goat anti-mouse HRP conjugated IgG from Jackson Labs., Cat. # 115-035-068) in TBST for 30 min-1 h at room temperature with constant agitation.
12. Wash the membrane 5 times in TBST for 10 min each.
13. Use an enhanced chemiluminescence detection method to detect the RhoA signal (e,g. ,SuperSignal West Dura Extended Duration Substrate, ThermoFisher)

Recipe for Western Blot Buffer (1 L)

1 M Tris pH 8.3	25 ml	(25 mM final)
Glycine	14.4 g	(192 mM final)
Methanol	150 ml	(15% final)
Distilled water to 1 L		

VII: Troubleshooting

Observation	Possible cause	Possible Remedy
No signal from the His-tagged control protein.	1. Storage of the stock control protein at concentrations that are too low (<0.33mg/ml). 2. Repeated freeze/thaw cycles of the reconstituted positive control stock protein. 3. Attempts to store the diluted stock at 4°C or frozen for future use. 4. Not following the western blot method detailed in Assay Protocol: STEP 4.	1. The kit supplies 10 µg of His-tagged RhoA , Rac1 and Cdc42 protein, these should be reconstituted to a 0.33 mg/ml stock solution and stored at -70°C (as 10 x 3 µl aliquots, see Table 2). Storage of the protein at lower concentrations will result in denaturation and precipitation of the protein and incorrect quantitations or no signal at all. 2. The stock protein must be aliquoted as described in Table 2. Repeated freeze thaws of the stock will result in denaturation and precipitation. 3. We recommend loading 20 ng of the positive control on the gel as a positive control and quantitation estimate for endogenous small G-protein (for 20 ng of recombinant protein, dilute one 3 µl aliquot of protein stock with 247 µl of Cell Lysis Buffer and then 250 µl of 2x Laemmli sample buffer; load 10 µl of this on the SDS gel). The diluted protein is unstable and will precipitate. Unused protein must be discarded. The Rho family proteins have a molecular weight of between 20-25 kDa; the His-tagged control proteins have a molecular weight of approximately 23-30 kDa. 4. Make sure that the western blot method described in Assay Protocol: STEP 4 is followed.
No difference in signal between GTPγS positive control and GDP negative control assay	1. Protein lysate concentrations were not equalized. 2. GDP requirements are higher for your cell line.	1. The absolute amount of protein in lysates can have a dramatic effect upon Rho family protein signal. It is therefore very important to have equal amounts of cell lysate protein in each reaction. See section V (E). 2. Some cell lines have very high levels of endogenous GTP and exchange of GDP requires addition of greater than the 1 mM GDP outlined in this manual. We recommend trying 10 mM GDP in these cases.
No detectable activation in the positive control (GTPγS) assay	1. STOP buffer not added to the reactions. 2. Leaving the lysates for >10 minutes before use.	1. Follow the instructions carefully, for example, STOP buffer must be added to the reaction or you will not get a positive pull-down signal. 2. GTPγS AND GDP loaded lysates should be used within 2-3 minutes after STOP buffer has been added.

VII: Troubleshooting (cont.)

Observation	Possible cause	Remedy
No detectable signal in the experimental samples	1. Control reaction not performed for GTPγS. His-tagged control protein not used during Western blot. 2. Insufficient cell lysate used 3. Lysates not processed rapidly at 4°C	1. Always run a GTPγS control to make sure the affinity beads are working and always run the recombinant His-tagged control protein to make sure that the Western blot antibody is working correctly. Once these controls are working you can go on to determine the likely cause of a lack of signal or a lack of activation in the experimental samples. 2. Titrate the protein amount used in the assay. We recommend 300-800 µg lysate, however, in some cases more lysate may be required. 3. Rho family proteins are still able to hydrolyze GTP during lysate preparation; hydrolysis is stopped only when the affinity beads are bound to the small G-protein. The temperature and speed of lysate preparation are therefore very important parameters in this assay .
Small G-protein activation signal does not change upon experimental activation stimulus.	1. Titration of affinity beads not performed. 2. Culture conditions have caused cells to become unresponsive to activators. 3. Selected activator may not work with your cell line. 4. Western blot is overexposed leading to inaccurate readings.	1. Make sure that your control GDP and GTPγS lanes give a clear positive and negative response; this indicates that the bead and cell lysate levels are in the correct linear range to detect differential activation states. This may require titrating bead and / or lysate levels. 2. Continuous overgrowing of a cell line can result in unresponsive cells. Swiss 3T3 cells should only be used for 10 passages and then discarded as their properties change if they are passaged longer than this (18). Cells seeded at low densities, grown for 3 days to 30-40% confluency, then serum starved by a serum-step down procedure often respond better than cells grown to higher densities. 3. Use a known activator (eg. Calpeptin for RhoA, EGF for Rac1 or Cdc42) to check the responsiveness of your cell line. A list of some activators are given in Appendix 2. Note that the cell line used for the activation assay is important as response to any given activator can vary considerably between cell lines. It may also be useful to examine actin morphology via rhodamine-phalloidin labeling of cells. (See Appendix 1). 4. As a general guideline, you should expose the film so that the small G-protein signal gives a grey band rather than a black band. Alternatively, a RhoA , Rac1 or Cdc42 G-LISA® Activation Assay Kit (Cat. # BK124, BK128 and BK127) can be used to obtain quantitative results within 3 h.

VIII: References

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Appendix 1: Observation of Actin Morphology By Rhodamine-Phalloidin Staining

Reagents needed

- Suitable growth media
- Calpeptin stock solution (20 mg/ml in PBS) for RhoA activation.
- Epidermal Growth Factor (EGF; 20 mg/ml stock) for Rac1 and Cdc42 activation.
- PBS solution pH 7.4 (150 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2PO_4, 1.47 mM KH_2PO_4)
- Rhodamine-phalloidin stock (14 µM in methanol, Cat. # PHDR1)
- Paraformaldehyde stock (6% stock in PBS, stored aliquoted at -20°C)

Method

Serum starvation for Swiss 3T3 cells and addition of growth factors

1. Swiss 3T3 cells are seeded at low density of 3 – 5 x 10^4 cells in DMEM plus 10% FCS on a 10 cm diameter plate containing two 13 mm diameter glass coverslips.
2. Once cells are 30-40% confluent (usually 3 days) the media is replaced with DMEM plus 1% FCS and cultured for 24 h.
3. The media is again replaced with DMEM without FCS and the cells are incubated for 16 - 20 h.
4. After serum starvation remove one coverslip and process for actin staining as described below.
5. For RhoA activation, add fresh calpeptin to the remaining cells to 100 µg/ml for 20 minutes.
6. For Rac1 activation, add EGF to 10 ng/ml for 2 minutes.
7. For Cdc42 activation, add EGF to 100 ng/ml for 2 minutes.
8. Remove the coverslip and process for actin staining as described below.

Actin Staining

1. Wash the cells once with PBS and fix for 20 min at room temperature in 3% paraformaldehyde diluted in PBS.
2. Wash the cells once for 30 s with PBS to remove excess fixative.
3. Incubate the cells with 0.2% Triton-X 100 in PBS for 5 min at room temperature to permeabilize cells.
4. Wash twice in PBS for 30 s each.
5. Incubate with 200 µl of 0.1 µg/ml Rhodamine-phalloidin for 30 min at room temperature in the dark.
6. Wash five times with PBS for 30 s each.
7. Invert the cells into mounting medium (eg. Polyvinyl alcohol mounting medium, Fluka Chemie) and allow the coverslip to set for 30 min.

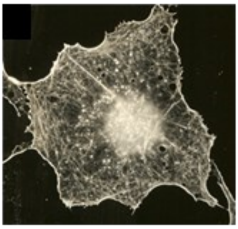
8. View actin filaments with a 63 – 100X oil immersion objective.

9. Examples of serum starved and calpeptin treated cells are shown in Figure 1.

NOTE: All the required reagents for fixing cells and staining F-actin can be found in the F-actin Visualization Kit (Cat. # BK005).

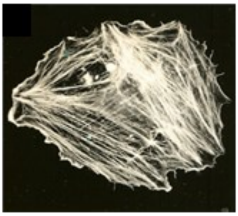
Figure 1. Rhodamine-Phalloidin Staining of the Actin Cytoskeleton in Serum Starved and Rho Family Activated Cells

A



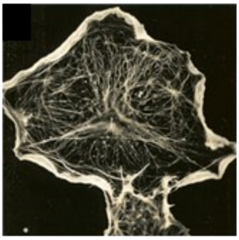
Serum Starved Actin Morphology:
Swiss 3T3 cells serum starved according to the method in this section prior to actin filament staining with rhodamine-phalloidin. In the absence of Rho family activation there is a notable paucity of actin filaments visible in the cell.

B



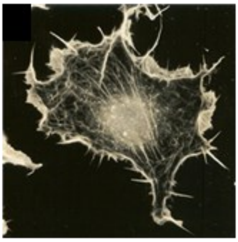
Rho Activated Actin Morphology:
Cells treated for 20 min with 100 µg/ml calpeptin after serum starvation and subsequently stained with rhodamine- phalloidin. Rho induced actin stress fibers are clearly visible.

C



Rac Activated Actin Morphology:
Cells treated for 2 min with 10 ng/ml EGF after serum starvation and subsequently stained with rhodamine phalloidin. Rac induced actin-rich lamellipodia and membrane ruffles are clearly visible.

D



Cdc42 Activated Actin Morphology:
Cells treated for 2 min with 100 ng/ml EGF after serum starvation and subsequently stained with rhodamine-phalloidin. Cdc42 induced actin-rich filopodia and microspikes are clearly visible.

Appendix 2: Known Rho Family Activators

Activator*	Treatment	Cell line used	Activated Protein	Response	Type of Assay Used	Ref.
Calpeptin (Cat. # CN01) (protease inhibitor, protein tyrosine phophatase inhibitor)	100 µg/ml	REF-52 fibroblasts & Swiss 3T3 cells adherent	Rho	Maximal activation after 5 to 10 min with extended activation time up to 30 min, decreasing thereafter to basal levels after 60 min	Actin morphology	1
Colchicine *(microtubule destabilizer)*	10 µg/ml	Swiss 3T3 cells, adherent or suspension	Rho	Maximal activation of 2-4 fold activation after 30 min	Rhotekin-RBD pull-down	2
Fibronectin *(extracellular matrix protein)*	Culture plate is coated with fibronectin	Swiss 3T3 cells	Rho	Biphasic regulation after plating cells on fibronection coated plates. Initial period of low RhoA activity (10 -20 min) followed by a 1-7 fold activation peaking at 60-90 min and then dropping to basal levels after 6 h	Rhotekin-RBD pull-down	2
Lysophospatidic acid (LPA)	1 µM	N1E-115 neuronal cells	Rho	Maximal activation of 3-5 fold after 3 min	Rho-kinase pull-down assay	3
Epidermal Growth Factor (EGF)	50 ng/ml	US7MG Human glio-blastoma	Rac	1.5 fold activation after 5 min with 2D cultures. 1.3 fold activation in 3D cultures	Rac G-LISA®	4
MCP-1	10 ng/ml	Murine alveolar macrophages	Rac	Maximum activation at 4h	Rac G-LISA®	5
Interleukin-3	5 ug/ml	Primary bone marrow derived mast cells	Rac	2.0 fold increase over control cells after 5 minutes	PAK-PBD pull-down assay	6
Tumor necrosis factor alpha (TNFα)	20 ng/ml	Swiss 3T3	Cdc42	Cdc42 specific activation after 10 minutes. Longer incubations resulted in Rac and Rho activation.	Actin morphology: filopodia formation	7
Tumor necrosis factor alpha (TNFα)	100 ng/ml	MEF cells	Cdc42	Filopodia formation increased rapidly and was greatest at 10 min after which filopodia decreased. PAK-PBD pull-down assays confirmed maximum Cdc42 activation of 3 fold after 10 minutes. Activation was maintained for several hours.	Actin morphology and PAK-PBD pull-down assay	8
Epidermal Growth Factor (EGF)	50 ng/ml	COS cells	Cdc42	Rapid activation upon exposure to growth factor, reaching a peak at approximately 10 min. Enhanced Cdc42 activation lasted at least 30 min in COS cells.	PAK-PBD pull-down assay	9

Appendix 2: Known Rho Family Activators (cont.)

References for Rho Family Activators

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Appendix 3: Protein Quantitation (with Precision Red Reagent)

Background

The Precision Red Advanced Protein Assay Reagent is a simple one step procedure that results in a red to purple/blue color change characterized by an increase in absorbance at 600 nm. The reagent is not supplied in this kit. It is sold separately as Cat. # ADV02. Precision Red Advanced Protein Assay Reagent is supplied in the G-LISA activation assays (Part# GL50).

The assay exhibits low variance in readings between different proteins of the same concentration and high reproducibility of the colorimetric response. This allows one to utilize a generally applicable standard curve (Fig. 1) for protein quantitation. The assay can also be performed in approximately 1-2 minutes. These properties are particularly valuable when applied to the labile lysates required for activation assays.

Quick Protein Concentration Method for 1 ml Cuvette (recommended)

- Add 20 µl of each lysate or Lysis Buffer into disposable 1 ml cuvettes.
- Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Cat# ADV02) to each cuvette.
- Incubate for 1 min at room temperature.
- Blank spectrophotometer with 1 ml of ADV02 plus 20 µl of Lysis Buffer at 600 nm.
- Read absorbance of lysate samples.
- Multiply the absorbance by 5 to obtain the protein concentration in mg/ml

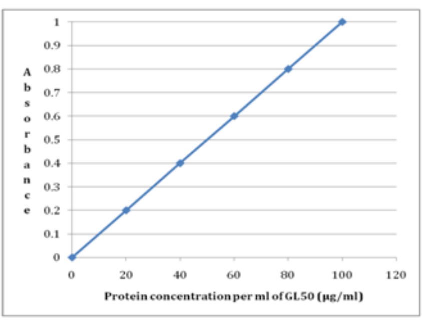


Fig. 1: Standard Curve for Protein Quantitation in a 1ml Cuvette

Legend: The standard curve shown in Fig. 1 represents the average absorbance reading of several common proteins (e.g., actin, BSA, casein) measured in a 1 ml cuvette format using 1 ml of ADV02 reagent. The protein reading pathlength for a cuvette is 1 cm. Linear range of this assay is 0.05 - 0.6.

<u>Example Calculation</u>

Assume a 20 µl sample of cell lysate added to 1 ml of ADV02 gives an absorbance reading of 0.1.

$$C = \frac{A}{\varepsilon l} = \frac{0.1}{10 \times 1} \times 50 = 0.5 \text{ mg/ml}$$

Where c = protein concentration (mg/ml), A = absorbance reading, l = pathlength (cm), ε = extinction coefficient ($[\text{mg/ml}]^{-1}$ cm^{-1}) and the multiplier of 50 is the dilution factor for the lysate in ADV02 (20 µl lysate in 1 ml ADV02).

Thus, for a 20 µl sample in 1 ml ADV02, the equation becomes C = A x 5

For a 10 µl sample in 1 ml ADV02, the equation becomes C = A x 10

Quick Protein Concentration Method for 96 Well Plate

- Add 10 µl of each lysate or Lysis Buffer into the well of a 96 well plate.
- Add 290 µl of Precision Red™ Advanced Protein Assay Reagent to each well.
- Incubate for 1 min at room temperature.
- Blank spectrophotometer with 290 µl of ADV02 plus 10 µl of Lysis Buffer at 600 nm.
- Read absorbance of lysate samples.
- Multiply the absorbance by 3.75 to obtain the protein concentration in mg/ml

96 Well Plate Method
The linear range of this assay is 0.05 - 0.4 and is recommended when lysates are below the linear range of the 1 ml cuvette method. The pathlength for 96 well plate readings is 0.8 cm, hence the equation is modified as shown in the example below:

Example Calculation for 96 Well Plate Measurement
Assume a 10 µl sample of cell lysate added to 290 µl of ADV02 gives an absorbance reading of 0.1

$$C = \frac{A}{\varepsilon l} = \frac{0.1}{10 \times 0.8} \times 30 = 0.375 \text{ mg/ml}$$

Where c = protein concentration (mg/ml), A = absorbance reading, l = pathlength (cm), ε = extinction coefficient ($[\text{mg/ml}]^{-1}$ cm^{-1}) and the multiplier of 30 is the dilution factor for the lysate in ADV02 (10 µl lysate in 290 µl ADV02).

Thus, for a 10 µl sample in 290 µl ADV02, the equation becomes C = A x 3.75

For a 5 µl sample in 295 µl ADV02, the equation becomes C = A x 7.5

NOTE: The protein concentrations generated by using the standardized protein curve (Fig.1) will generate approximate lysate concentrations. Data will be highly reproducible from lysate to lysate and will generate excellent values for relative concentrations of a series of lysates. It should be noted for activation assays, the relative protein concentration between experimental extracts is far more important than the absolute protein quantitation. However, if desired, one can create a standard curve using BSA or IgG protein standards for each experiment. The standard curve should be performed prior to lysate preparations due to the labile nature of the lysates.

Appendix 4: Total RhoA ELISA Method

Normalization of Active RhoA against Total RhoA is required for comparison of RhoA activity between samples (1-4). Normalization of active RhoA signal is particularly important in studies that involve prolonged exposure of cells to conditions that might affect RhoA pathways, e.g., transfections or drug studies.

In order to avoid the need to perform poorly reproducible and semi-quantitative western blot analysis, Cytoskeleton Inc. has developed an ELISA assay to allow rapid and quantitative determination of Total RhoA (Cat # BK150). It is generally accepted that active RhoA comprises between 0.5-5% of total RhoA in normal cellular transduction processes (5).

Method

Swiss 3T3 cells were grown to 30% confluency in DMEM media plus 10% FCS. They were then serum starved for 48h. Half of the cells were treated with 0.1 mg/ml calpeptin (Cat# CN01) for 30 minutes to activate RhoA. The other half were not treated. All cells were subsequently lysed in Cell Lysis Buffer (Part# CLB01) and frozen as 12.5 µg aliquots ready for analysis by ELISA (Fig. 1) or 900 µl aliquots ready for analysis by the RhoA pull-down assay (data not shown).

Results

The Rho ELISA data in Fig.1 show 12.5 µg lysate contained 12 ng Total RhoA in calpeptin treated cells and 11 ng Total RhoA in serum starved cells.

RhoA pull-down assays showed that 450 µg of lysate contained 13.3 ng active RhoA (0.37ng in 12.5 µg lysate) in calpeptin treated cells and 3.6 ng of Active RhoA (0.1ng in 12.5 µg lysate) in serum starved cells (data not shown).

Data Analysis

The fold activation of calpeptin treated cells can be determined using the simple formula given below:

$$\frac{\textbf{Pull-down value (ng)}}{\textbf{ELISA value (ng)}} \times 100 = \textbf{normalized \% Active RhoA in a given lysate}$$

For serum starved lysates

$$\frac{0.10}{11} \times 100 = \textbf{0.91\% Active RhoA in untreated cells}$$

-

For calpeptin treated lysates

$$\frac{0.37}{12} \times 100 = \textbf{3.1\% Active RhoA in stimulated cells}$$

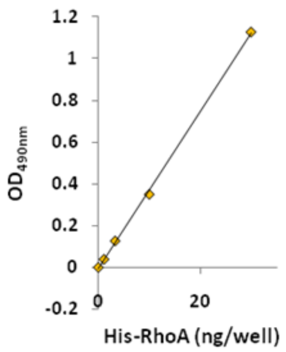
Thus, the normalized fold activation for calpeptin treated cells compared to untreated serum starved cells is **3.4 fold**

Appendix 4: Total RhoA ELISA Method (cont.)

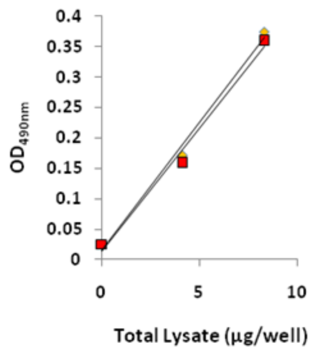
It can be seen that Total RhoA is very similar in the calpeptin and serum starved samples. This is to be expected with lysates from cells that have only been briefly treated with an activator. In this case the purpose of the normalization is simply to confirm that equal amounts of lysate have been analysed. In transfection experiments or more prolonged drug treatments, it cannot be assumed that Total Rho levels will be identical in equivalent amounts of cell lysate.

Figure 1: Determination of Total RhoA by ELISA

2A: Standard RhoA curve



2B: ELISA of calpeptin and serum starved lysates



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Appendix 5: Processing Tissue Samples for Pull-Down Assays

Tissue lysates can be used in pull-down assays (1). Recommendations regarding tissue lysates are given below;

1) Rho family GTPases are labile proteins that will hydrolyze bound GTP during sample handling. Tissues should therefore be processed quickly and at 4°C if possible. Tissues should be processed immediately using 4°C buffers or cut into small chunks (3-5 mm diameter), snap frozen in liquid nitrogen and stored at –70°C for later processing.
2) Tissues can be extracted using a micro-pestle on ice. Homogenates should be clarified by a 1 minute centrifugation at 4°C. Lysates can be used immediately in an activation assay or snap frozen in "experiment-sized" volumes. The Activation Assay uses approximately 300-800 µg of total protein per assay; this translates to 600-1600 µl of a 0.5 mg/ml cell lysate. We recommend duplicate samples per time-point or condition, therefore 1.2– 3.2 ml aliquots are recommended for snap freezing.
3) When possible tissues should be extracted in Cell Lysis Buffer (Part# CLB01) as this is the recommended buffer for pull-down assays.
4) It is recommended that lysis buffer be supplemented with protease inhibitors and phosphatase inhibitors. Recommended inhibitors include; Cytoskeleton protease inhibitor cocktail (Cat# PIC02), sodium fluoride (50 mM final), sodium pyrophosphate (20 mM final), p-Nitrophenyl phosphate (1 mM final) and microcystin LR (1 µM final).
5) A final lysate protein concentration of 0.5 mg/ml is recommended.

Reference

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