RHO ACTIVATION ASSAY BIOCHEM KIT

Cat. # BK036

ORDERING INFORMATION

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MANUAL CONTENTS

Section I: Introduction

Rho Activation Assay:

The Rho family of small GTPases consists of at least 14 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). Understanding the mechanisms that regulate activation / inactivation of the GTPases is of obvious biological significance and is a subject of intense investigation. The fact that many Rho family effector proteins will specifically recognize the GTP-bound form of the protein (13) has been exploited experimentally to develop a powerful affinity purification assay that monitors Rho protein activation (14). The 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 "pulldown" GTP-Rho complexed with rhotekin-RBD beads. This assay provides a simple means of analyzing cellular Rho activities in a variety of systems (see Rho Activation Assay Kit citations on Appendix 3). The amount of activated Rho is determined by a Western blot using a Rho specific antibody. A typical Rho pulldown assay using GTP and GDP loaded human platelet extract or Swiss 3T3 cells extracts is shown in Figure 1.

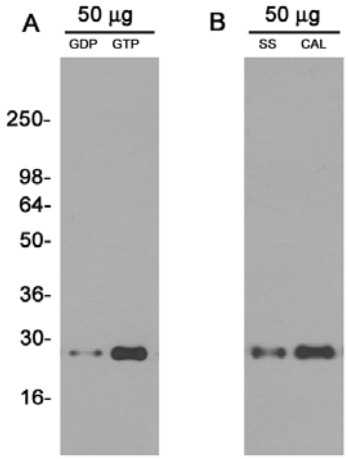

Figure 1. Rhotekin-RBD bead pulldown Assays. *A. Extract (300 µg) from human platelet cells was loaded with GTPγS (GTP lane) or GDP (GDP lane) using the method described in Section VI: Control Reactions. B. Extract (300 µg) from serum starved (SS) and subsequent calpeptin (CAL) treated Swiss 3T3 cells. All extracts were incubated with 50 µg of rhotekin-RBD beads and processed as described in Section VI: Pulldown Assay. All bead samples were resuspended in 10 ul 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.*

Section II: Purchaser Notification

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Section III: Kit Contents

This kit contains enough reagents for approximately 80 pulldown assays. There is sufficient Rho antibody for 200 ml working strength primary antibody solution.

Table 1: Kit Contents

Reagent	Cat. # Part #	Quantity	Description
Rhotekin RBD beads	RT02	2 tubes	Lyophilized. 2 mg of protein per tubes bound to colored sepharose beads.
Anti-RhoA monoclonal antibody	ARH03	2 tubes	Lyophilized. 50 µg protein per tube.
His-RhoA control protein	RHWT	1 tube	Lyophilized. 10 µg of protein (~30 kDa) as a Western blot standard.
Cell Lysis Buffer	CLB01	1 bottle	Lyophilized. 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, and 2% Igepal when reconstituted.
Wash Buffer	WB01	1 bottle	Lyophilized. 25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl when reconstituted.
Loading Buffer	LB01	1 tube	Liquid. 150 mM EDTA solution.
STOP Buffer	STP01	1 tube	Liquid. 600 mM $MgCl_2$ solution.
GTPγS stock: (non-hydrolysable GTP analog)	BS01	1 tube	Lyophilized. 20 mM solution, when reconstituted.
GDP stock	GDP01	1 tube	Lyophilized. 100 mM solution, when reconstituted.
Protease inhibitor cocktail	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.
Anhydrous DMSO	DMSO	1 tube	Solvent for protease inhibitor cocktail.

Required reagents/components that are not supplied:

Laemmli sample buffer.
Polyacrylamide gels (12% or 4-20% gradient gels).
SDS-PAGE buffers.
Western blot buffers.
Protein transfer membrane (PVDF or Nitrocellulose).
Secondary antibody (eg. Goat **anti-mouse** HRP conjugated IgG, Jackson Labs. Cat. # 115-035-068).
Chemiluminescence based detection system. (eg. ECL Advanced Western Blotting Detection Kit GE Healthcare)

Section 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 follows:

Table 2: Reconstitution and Storage of Components

Component	**Reconstitution**	**Storage**
Rhotekin-RBD protein beads	Reconstitute each tube in 600 µl distilled water. Aliquot into 6 x 100 µl volumes (15 µl of beads = 50 µg protein, under these conditions 100 µl is sufficient for 6 assays). **Snap freeze in liquid nitrogen.**	Store lyophilized protein desiccated at 4°C. Stable for 6 months. Store resuspended protein frozen at -70°C. Stable for 1 year.
Anti-RhoA monoclonal antibody	Resuspend each tube in 200 µl of PBS. Use at 1:500 dilution.	Store at 4°C. Stable for 6 months.
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 lyophilized protein desiccated at 4°C. Stable for 6 months. Store resuspended protein at -70°C for 6 months.
Cell Lysis Buffer	Reconstitute in 100 ml of sterile distilled water and store at 4°C. **NOTE**: This solution may take 5-10 min to resuspend, use a 10 ml pipette to thoroughly resuspend the buffer. To keep long term stocks of this buffer; aliquot the solution into 20 x 5 ml volumes and store at -20°C.	Store lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at 4°C for 3 months.
Wash Buffer	Reconstitute each in 100 ml of sterile distilled water and store at 4°C. To keep long term stocks of this buffer; aliquot the solution into 5 x 20 ml volumes and store at -20°C.	Store lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at 4°C for 3 months.
Loading Buffer	None required.	Store at 4°C.
STOP Buffer	None required.	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 lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at -70°C Stable for 6 months.
GDP stock	Reconstitute in 50 µl of sterile distilled water. Aliquot into 5 x 10 µl volumes, snap freeze in liquid nitrogen.	Store lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at -70°C Stable for 6 months.
Protease inhibitor cocktail	Reconstitute in 1 ml of DMSO (100x solution).	Store lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at -20°C Stable for 6 months.

Section V: Important Technical Notes

A. Updated Manual (Version 4.2 and up) Review

The following updates from the previous Version should be noted:

1) The rhotekin-RBD beads (Cat. # RT02) have been optimized to give a much higher affinity for active RhoA and can now be used at lower bead concentrations than previously recommended (see Figure 2). In general, the 50 µg bead pull-down will yield optimal results. Under these conditions the 4 mg of rhotekin-RBD beads supplied in the kit are sufficient for 80 assays. We do however recommend a bead titration to determine your optimal pull-down conditions (as shown in Figure 2).

2) The BK036 manual Version 4.2 has been updated to give more extensive details on how to process cell lysates prior to performing the pull-down. The new manual reflects a strong preference towards freezing "experiment-sized" aliquots of lysates prior to performing the assay. Snap freezing aliquots of lysate in liquid nitrogen has many advantages:
   i) The level of small G-protein activation is identical between fresh, rapidly processed lysates and lysates that have been snap frozen in liquid nitrogen and stored at -70°C prior to thawing and use in an activation assay. By freezing multiple aliquots of any given lysate one can perform multiple assays on a given lysate to compare results from different experiments.
   ii) Rapid processing and freezing of lysate allows more time to quantitate protein concentration in a given lysate without allowing the whole lysate sample to sit on ice for excessively long periods of time. Remember that lysates are relatively unstable prior to addition of the beads and excessive processing time will result in GTP hydrolysis by the small G-protein and consequent loss of signal.
   iii) Time-courses can be performed sequentially and each time-point can be snap frozen prior to performing the second time-point experiment. This makes the taking of time-points a very simple process and does not result in excessive processing time for early time-point lysates.

   NOTE: Lysate samples MUST be snap frozen in liquid nitrogen to maintain the correct activation level of small G-protein. Slower freezing methods are not acceptable.

3) The Western blot procedure for small-G protein transfer has been optimized (see Section VI: STEP 4). This protocol optimizes the transfer of small G-proteins and recommends the use of the anti-RhoA antibody at a 1:500 dilution (previously at 1:750).

4) Protease Inhibitor Cocktail should be added to the Cell Lysis Buffer (1x final conc.) immediately prior to use. **NOTE:** The addition of 0.5% Sodium deoxycholate and 0.1% SDS to Cell Lysis Buffer can improve cell lysis and does not interfere with the rhotekin-RBD binding assay. In some cell lines these detergents can cause nuclear lysis resulting in viscous cell extracts.

Section V: Important Technical Notes, continued

B. Growth and Treatment of Cell Lines

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

Adherent cells should be ready at 40 – 60% confluence or for non-adherent cells at approximately 3×10^5 cells per ml. Briefly, cells are seeded at 0.5×10^4 per ml (approximately 5×10^4 cells per 10 cm dish) and grown for 3 days. Serum starvation or other treatment will be performed when they are 40 - 50% confluent (See Appendix 1).

When possible, the untreated samples should have cellular levels of Rho activity in a "controlled state". For example, when looking for Rho activation the "controlled state" cells could be serum starved. Serum starvation will inactivate cellular Rho and lead to a much greater response to a given Rho 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. 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. In general, cells grown to >80% confluence should not be used for Rho 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 vs. the "responsive state" cells by TRITC-phalloidin staining of actin filaments. A detailed method for actin staining is given in Appendix 1. Rho activation causes the formation of characteristic actin stress fibers (15).

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.

Section V: Important Technical Notes, continued

C. Timing and Intensity of RhoA Activation

Upon stimulation, Rho proteins are generally activated very rapidly and transiently, maximal activation ranges from 30 s to 30 min and declines thereafter to basal levels. For potent activators such as calpeptin or LPA, the intensity of maximal RhoA 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 activation peak. 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 min (this time course is also recommended for RhoA inactivation studies).

In practical terms the timed experiment should 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 in -70^{o}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 is a labile entity, the bound GTP is susceptible to hydrolysis by Rho-GAPs during and after cell lysis, resulting in Rho inactivation. Rapid processing at 4°C is essential for accurate and reproducible results. The following guidelines are useful for rapid washing of cells.

Washing

1) Retrieve culture dish from incubator, immediately aspirate out all of the media and place firmly on ice.
2) Immediately rinse cells with an appropriate volume of ice cold PBS to remove serum proteins (see Table 3 for recommended wash volumes).
3) Aspirate off all residual PBS buffer. This is essential so that the Cell Lysis Buffer is not diluted. Correct aspiration requires that the culture dish is placed at an angle on ice for 1 min to allow excess PBS to collect in the vessel for complete removal.

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 Cultures

Culture Vessel	**Vessel surface area (cm^2)**	**Volume of PBS wash (ml)**	**Volume of Lysis Buffer (µl)**
35 mm dish	8	2.0	100
60 mm dish	21	3.0	150
100 mm dish	56	10.0	400
150 mm dish	148	15.0	1200
6-well cluster plate	9.5 / well	3.0	100
12-well cluster plate	4 / well	1.5	60
T-25 Flask	25	4.0	160
T-75 Flask	75	10.0	800
T-150 Flask	150	15.0	1200

Section V: Important Technical Notes, continued

Cell Lysis, continued

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

1) Work quickly.
2) Keep solutions and lysates embedded 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 sample should also be kept for Western blot quantitation of total RhoA per sample. The lysates **must** be snap frozen in liquid nitrogen and stored at -70°C. Lysates should be stored at -70°C for no longer than 30 days.
4) Thawing of cell lysates prior to the use in the pulldown assay should be in a room temperature water bath, followed by rapid transfer to ice and immediate use in the assay.

E. Protein Concentration and Quantitation of Total RhoA:

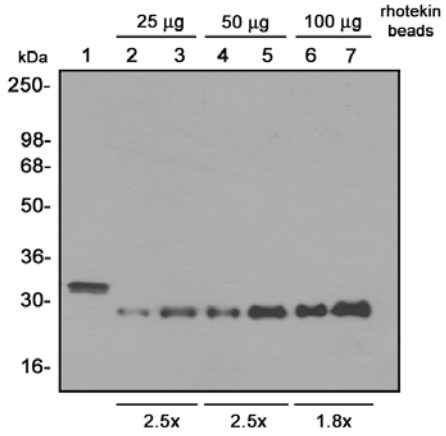
Equal protein concentration in all samples is a prerequisite for accurate comparison between samples in Rho activation assays. Cell extracts should be equalized with ice cold Cell Lysis Buffer to give identical protein concentrations. For example, cell lysates of protein concentrations ranging from 0.5–1.3 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 make sure that equal amounts of total RhoA are assayed, we recommend including samples of total lysate from experimental samples in the Western blot. Samples of 20 – 50 µg total cell lysate per sample should be sufficient to detect total RhoA.

F. Assay Linearity

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

1) **Bead Titration:** Rhotekin-RBD will bind to Rho-GDP with a much lower affinity than Rho-GTP. If too many rhotekin-RBD beads are added to the pulldown assay there will be significant binding to inactive (GDP-bound) RhoA. The result of this will be an underestimate of RhoA activation (see Figure 2). For this reason we highly recommend performing a bead titration to determine optimal conditions for any given RhoA 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.



Section V: Important Technical Notes, continued

Bead Titration, continued

Figure 2. Rhotekin-RBD Bead Titration on Serum Starved and

Calpeptin Treated Swiss 3T3 Cell Extracts. *Previously frozen extract (300 µg at 0.5 mg/ml) made from serum starved (samples 2, 4 and 6) or calpeptin treated (samples 3, 5 and 7) Swiss 3T3 cells (see Appendix 1) were incubated with increasing amounts of rhotekin-RBD beads (25, 50 or 100 µg) and processed as described in Section VI:STEP 3: Pulldown 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 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 rhotekin-RBD 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.

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.

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Section VI: Assay Protocol

STEP 1: Control Reactions

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

1. **Whole Cell Lysate Protein:**
   Total RhoA present in each sample should be determined by Western quantitation. Usually 20 – 50 µg of cell lysate will result in a good signal. The total RhoA should not differ by more than 10-20% between samples.

2. **Positive Cellular Protein Control:**
   Total cell lysate (300 – 800 ug) should be loaded with GTPγS as a positive control for the pulldown assay. The following reaction details how to load endogenous RhoA with the non-hydrolysable GTP analog (GTPγS), this is an excellent substrate for rhotekin-RBD beads and should result in a strong positive signal in a pulldown assay.

   a. Perform GTP loading on 300 – 800 µg of cell lysate by adding 1/10th volume of Loading Buffer.
   b. Immediately add 1/100th volume of GTPγS (200 µM final concentration). Under these conditions 5 - 10% of the Rho protein will load with non-hydrolysable GTPγS and will be "pulled down" with the rhotekin-RBD beads in the assay (see Figure 1).
   c. Incubate the control sample at 30°C 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.
   e. Use this sample in a pulldown 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 RhoA with GDP will inactivate Rho and this will bind very poorly to rhotekin-RBD beads.

4. **His-RhoA Protein Control:**
   The kit supplies 10 µg of His-RhoA control protein; this 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 will result in denaturation, precipitation of the protein and incorrect quantitations or no signal in the western blot. We recommend that 20 ng of His-RhoA control protein be run on the gel as a positive control and as a quantitation estimate for endogenous Rho. The Rho family proteins have a molecular weight of between 20-25 kDa; the His-tagged control protein has a molecular weight of approximately 30 kDa.

Section VI: Rho Activation Assay Protocol, Continued

STEP 2: Lysate Collection

We strongly recommend that you snap freeze the 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 lysate processing are given below:

1. Grow cells in appropriate culture conditions. It is important to keep cells in a "controlled state" prior to Rho activation, see Section V: B.

2. Treat cells with Rho 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.

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 Section V: D Table 3, 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 rpm, 4°C for 2 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 Western quantitation of total RhoA.

11. Aliquot and snap freeze the remaining cell lysates in liquid nitrogen. Store at -70°C for future use. It is recommended to aliquot 1-3 ml of lysate per tube (this should be sufficient for duplicate assays of 300 – 800 µg lysate per assay).

12. Measure lysate protein concentrations. We recommend using Precision Red Advanced Protein Assay (Cat. # ADV02) for quantitations:
    a. Add 1 ml of Precision Red Advanced Protein Assay Reagent (ADV02) to each disposable 1 ml cuvette
    b. Add 20 µl of each lysate or lysis buffer into cuvettes.
    c. Incubate for 1 min at room temperature.
    d. Blank spectrophotometer with the Cell Lysis Buffer at 600 nm.
    e. Read absorbance of lysates samples.
    f. Multiply the absorbance by 5 to obtain the protein concentration in mg/ml.

Section VI: Rho Activation Assay Protocol, Continued

13. Calculate how to equalize the cell extracts with ice cold Cell 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 be 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:

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

    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 pulldown assays simpler. Be aware of the length of time cell lysates stay on ice (should not exceed 10 min), since Rho-GTP hydrolysis will occur.

Section VI: Rho Activation Assay Protocol, Continued

STEP 3: Pulldown 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 beads from your bead titration test (see Section V.F.1 and Figure 2).

   NOTE: In general, a 50 µg (15 µl) bead pulldown will yield optimal results. Under these conditions the 4 mg of rhotekin-RBD beads supplied in the kit are sufficient for 80 assays. We do however recommend a bead titration (25, 50 & 100 µg) to determine optimal pulldown conditions.

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

4. Pellet the rhotekin-RBD 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 rhotekin-RBD 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 whole sample including the beads can be loaded onto the SDS gel if necessary. It is recommended that the necessary control samples be run on each gel.

Section VI: Rho Activation Assay Protocol, Continued

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. Equilibrate the gel in Western blot buffer (25 mM Tris, 192 mM glycine, 15% methanol, pH 8.3) for 15 min at room temperature prior to electro-blotting.

3. Transfer the protein to a PVDF membrane for 1 h at 350 mA.

4. Wash the membrane once with TBS (20 mM Tris-HCl pH 8.0, 50 mM NaCl).

5. Block the membrane surface with 5% nonfat-dry milk in TBST (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.05% Tween 20) for 30 min at room temperature with constant agitation.

6. Incubate the membrane with a 1: 500 dilution of anti-RhoA antibody (Cat. # ARH03, provided with kit) diluted in TBST (no blocking agent) for 1 h at room temperature or overnight at 4°C with constant agitation.

7. Wash the membrane three times in TBST for 10 min each.

8. 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.

9. Wash the membrane 5 times in TBST for 10 min each.

10. Use an enhanced chemiluminescence detection method to detect the RhoA signal (eg. ECL Advanced Western Blotting Detection Kit GE Healthcare).

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		

Section VII: Troubleshooting

1. No signal from the His-tagged RhoA control protein.

The kit supplies 10 µg of His-RhoA protein, this should 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 will result in denaturation and precipitation of the protein and incorrect quantitations or no signal at all. We recommend loading 20 ng of this positive control on the gel as a quantitation estimate for endogenous Rho (for 20 ng of recombinant protein, dilute one 3 µl aliquot of protein stock with 250 µl of Milli-Q water and then 250 µl of 2x Laemmli sample buffer; load 10 µl of this on the SDS gel). The Rho family proteins have a molecular weight of between 20-25 kDa; the His-tagged control protein has a molecular weight of approximately 30 kDa.

2. No difference in signal between GTPγS positive control and GDP negative control assay.

a) Equalize the protein lysate concentration.

The absolute amount of protein in lysates can have a dramatic effect upon RhoA signal. It is therefore very important to have equal amounts of cell lysate protein in each reaction. Any difference in total protein concentration (>10% between samples) will result in unreliable data. Protein quantitations should be carried out prior to bead addition but this should take no longer than 10 min. Use the Precision Red Protein Assay reagent (Cat. # ADV02) from Cytoskeleton Inc. for a 1 min protein concentration assay.

b) Titration of Rhotekin-RBD beads. The amount of rhotekin-RBD protein beads that will result in a good differential between GTP and GDP-bound RhoA must be determined from a bead titration experiment. In cases where there is a high signal in both GTPγS and GDP lanes, using half the amount of rhotekin-RBD beads will often result in a better differential signal.

c) Titrate Nucleotide addition

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.

d) Lysis Buffer Composition

Addition of 0.5% Sodium deoxycholate, 200 mM NaCl and 0.1% SDS to 1X Cell Lysis Binding buffer can give a better differential signal between GDP and GTPγS samples, probably due to an increased stringency of rhotekin-RBD binding. In some cell lines these detergents cause nuclear lysis resulting in viscous cell extracts and difficulty in handling the samples.

3. No detectable RhoA activation in the positive control (GTPγS) assay.

Follow the instructions carefully, for example, STOP buffer must be added to the reaction or you will not get a RhoA signal.

4. No detectable signal in the experimental samples.

a) Always run a GTPγS control to make sure the rhotekin-RBD beads are working and always run the recombinant His-RhoA control protein to make sure that the Western blot / RhoA 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.

Section VII: Troubleshooting, continued

b) A lack of signal may be due to insufficient cell lysate being used. Titrate the protein amount used in the assay.

c) RhoA is still able to hydrolyze GTP during lysate preparation; hydrolysis is stopped only when the rhotekin-RBD beads are bound to RhoA-GTP. The temperature and speed of lysate preparation are therefore very important parameters in this assay. ALWAYS process lysates at 4ºC and proceed as rapidly as possible.

5. RhoA activation signal does not change upon experimental activation stimulus

a) 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 RhoA activation states. This may require titrating bead and / or lysate levels.

b) In some cases culture conditions have caused the cells to become unresponsive to RhoA activators, this can be a major obstacle to obtaining meaningful results. For example, continuous overgrowing of a cell line can result in unresponsive cells. It has been reported that Swiss 3T3 cells should only be used for 10 passages and then discarded as their properties change if they are passaged longer than this (17). It is the experience of scientists at Cytoskeleton Inc. that 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. See Appendix 1 for a cell culture protocol.

   i. It is always a good idea to use a known RhoA activator (eg. Calpeptin) or inhibitor (eg. Cell permeable C3 transferase, Cat. # CT04) to check the responsiveness of your cell line. A list of some RhoA activators are given in Appendix 2. It will be noted that Appendix 2 gives the cell line used for the activation assay, this is important as response to any given activator can vary considerably between cell lines.

   ii. Because the morphological changes produced by RhoA activation result in actin stress fiber formation, it is useful to examine the morphology of the cells in response to serum starvation and a RhoA stimulator. Actin morphology can be examined by TRITC-phalloidin labeling of cells (see Appendix 1). The serum starved cells should have very few actin stress fibers while stimulated cells should have a large number of stress fibers (see Figure 3, Appendix 1).

c) The linear range of X-ray film is fairly narrow and overexposure of blots can result in inaccurate readings. Remember, a good RhoA activation signal will be 2 – 6 fold higher than unstimulated RhoA signal and care is needed to accurately measure these relatively small increases. As a general guideline, you should expose the film so that the RhoA signal gives a grey band rather than a black band.
Alternatively, the RhoA G-LISA™ Activation Assay Kit (Cat. # BK124) can be used to obtain quantitative results within 3 h.

Section VIII: References

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Section IX: Related Products:

PRODUCT (Cat. #)	DESCRIPTION	QUANTITY
BK121*	G-LISA™ for RhoA Activation Assay (Luminescence based)	96 assays
BK124*	G-LISA™ for RhoA Activation Assay (Absorbance based)	96 assays
BK125*	G-LISA™ for Rac1,2,3 Activation Assay (Absorbance based)	96 assays
BK126*	G-LISA™ for Rac1 Specific Activation Assay (Luminescence based)	96 assays
BK127*	G-LISA™ for Cdc42 Activation Assay (Absorbance based)	96 assays
BK128*	G-LISA™ for Rac1 Activation Assay (Absorbance based)	96 assays
BK129	G-LISA™ for RalA Activation Assay (Absorbance based)	96 assays
BK150	Total RhoA ELISA	96 assays
BK008	Ras Activation Assay Biochem Kit™ (Pull down assay)	25 assays
BK034	Cdc42 Activation Assay Biochem™ Kit (Pull down assay)	25 assays
BK035	Rac1 Activation Assay Biochem Kit™ (Pull down assay)	25 assays
BK100	Rho GEF exchange assay (Fluorimetric)	30-100 assays
BK105	RhoGAP assay (Colorimetric)	30-100 assays
BK005	F-Actin Visualization Biochem Kit™ *(for rhodamine phalloidin staining of actin filaments in fixed cells)*	300 – 1000 assays
GE01-A GE01-C	Human Dbs protein (His-tagged)	1 x 50 µg 3 x 50 µg
RT02-A RT02-B	Rhotekin-RBD beads	2 x 500 µg 10 x 500 µg
PAK02-A PAK02-B	PAK-PBD beads	1 x 250 µg 4 x 250 µg
R6301-A R6301-C	His tagged RhoA(63L) protein	1 x 20 µg 4 x 20 µg
ADV02	Protein quantitation assay kit	1 x 500 ml

* Patent# 7,763,418 B2

Appendix 1: Serum Starving Cells and F-Actin Visualization

Reagents needed

1. Suitable growth media
2. Calpeptin stock solution (20 mg/ml in PBS)
3. PBS solution pH 7.4 (150 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2PO_4, 1.47 mM KH_2PO_4)
4. TRITC-phalloidin stock (14 mM in methanol, Cat. # PHDR1)
5. Paraformaldehyde stock (6% stock in PBS, stored aliquoted at -20°C)

Method

Serum starvation 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. Add fresh calpeptin to the remaining cells to 100 µg/ml for 10 min.
6. 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 TRITC-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 3.

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

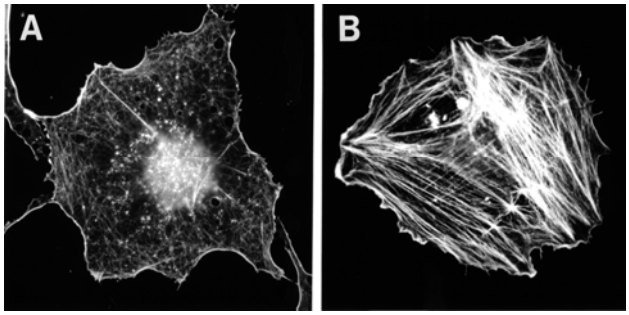

Figure 3. Rhodamine Phalloidin Staining of the Actin Cytoskeleton in Serum Starved and Calpeptin Treated Cells. *A) Swiss 3T3 cells serum starved for 16 h prior to actin filament staining with TRITC-phalloidin. B) Cells treated for 10 min with 100 µg/ml calpeptin after serum starvation and subsequently stained with TRITC-phalloidin.*

Appendix 2: Examples of Known RhoA activators

Activator*	Treatment	Cell Line	Response	Type of Assay	Reference
Colchicine *(microtubule destabilizer)*	10 µg/ml.	Swiss 3T3 cells, adherent or suspension.	Maximal activation of 2-4 fold activation after 30 min.	Rhotekin-RBD pulldown.	14
Nocodazole *(microtubule destabilizer)*	10 µM.	MG63 human osteosarcoma cells & HeLa cells.	Maximal activation of 2-3 fold activation after 30 min.	Actin morphology, rhotekin-RBD pulldown.	16, 17
Vinblastine *(microtubule destabilizer)*	50 µM.	MG63 human osteosarcoma cells.	Maximal activation of 2-4 fold activation after 30 min.	Actin morphology.	16
Cytochalasin D *(actin filament destabilizer)*	0.5 µg/ml.	Swiss 3T3 cells, adherent or suspension.	Maximal activation of 1-2 fold after 60 min.	Rhotekin-RBD pulldown.	14
Spingosine -1-phosphate *(serum lipid & G-coupled receptor agonist)*	1 µg/ml.	Swiss 3T3 cells, adherent or suspension.	Maximal activation of 1-2 fold after 2 min for 3T3 cells and 20 min for HUVEC cells.	Rhotekin-RBD pulldown.	14, 18
Serum	5 - 10%.	Swiss 3T3 cells, adherent or suspension.	Maximal activation of 2-6 fold (10%) and 1-2 fold (5%) after 1-5 min.	Rhotekin-RBD pulldown.	14
Lysophospatidic acid (LPA) *(serum lipid & G-coupled receptor agonist)*	1 µg/ml.	Swiss 3T3 cells, adherent & suspension.	Maximal activation of 2-6 fold after 1 min then dropping to basal after 30 min.	Rhotekin-RBD pulldown.	14
Lysophospatidic acid (LPA) *(serum lipid & G-coupled receptor agonist)*	1 µM.	N1E-115 neuronal cells.	Maximal activation of 3-5 fold after 3 min.	Rho-kinase pull down assay.	19
Fibronectin *(extracellular matrix protein)*	Culture plate is coated with fibronectin.	Swiss 3T3 cells.	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 minutes and then dropping to basal levels after 6 h.	Rhotekin-RBD pulldown.	14
Bombesin	10 nM.	Swiss 3T3 cells.	Maximal activation of 2-3 fold after 1 min which is sustained for at least 30 min.	Actin morphology.	1
Thrombin *(protease)*	10 nM.	HUVEC human venous endothelial primary cells (fifth passage cells).	Maximal activation of 14 fold after 2 min, dropping to basal levels after 30 min.	Rhotekin-RBD pulldown.	18

* In most cases activation of RhoA is performed on serum starved cells, see references for details.

Appendix 3: Citations of the Rho Activation Assay Kit (Cat. # BK036)

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