V3.1

G-LISA® RhoA Activation Assay Biochem Kit™ (Luminescence Based)

Cat. # BK121

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Manual Contents

I: Introduction

Background

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). In common with all other small GTPases, the Rho proteins act as molecular switches that transmit cellular signals through an array of effector proteins. This family is involved in a wide range of cellular responses, including cytoskeletal reorganization (2-3), regulation of transcription (4), cell migration (5), cellular transformation and metastasis (6).

The Rho switch operates by alternating between an active, GTP-bound state and an inactive, GDP-bound state. 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 Rho family effector proteins preferentially recognize the GTP bound form of the protein (7) has been exploited experimentally to develop a powerful affinity purification assay that monitors Rho protein activation (8).

Traditionally, this assay has been performed using a pull-down method, wherein the Rho-GTP-binding domain (RBD) of a Rho effector is coupled to agarose beads, allowing affinity based detection of the active Rho in biological samples (8). This method suffers from several drawbacks such as being time consuming, requiring large amounts of total cellular protein, being limited in the number of samples that can be handled simultaneously and yielding only semi-quantitative results.

The Rho G-LISA® Advantage

With the new G-LISA® kit (patent# 7,763,418 B2) you can now measure Rho activation from cell and tissue samples in less than 3 h. G-LISA® requires only 1-5% of the material needed for a conventional pull-down assay. You will also be able to handle large sample numbers and generate quantitative results. The G-LISA® advantages are summarized in Table 1.

Table 1: The G-LISA® Advantage

	Traditional pull-down	G-LISA®
Assay Time	10-12 h (2 days)	<3 h
Cell material per assay	1-2 mg protein (100 mm plate)	10-50 µg protein (12-well plate)
*Lysate clarification needed**	Yes	No
Sample handling	Up to 10 samples	Up to 96 samples (or more)
*Quantitative Data***	Semi	Yes
High throughput compatible	No	Yes

* Clarification is still recommended for low sample numbers. HTS applications that omit clarification have been developed.

** Numerical readouts and fewer sample handling steps make this assay more quantitative.

Assay Principle

The RhoA G-LISA® kit contains a Rho-GTP-binding protein linked to the wells of a 96 well plate. Active, GTP-bound Rho in cell/tissue lysates will bind to the wells while inactive GDP-bound Rho is removed during washing steps. The bound active RhoA is detected with a RhoA specific antibody and chemiluminescence. The degree of RhoA activation is determined by comparing readings from activated cell lysates versus non-activated cell lysates. Inactivation of RhoA is generally achieved in tissue culture by a serum starvation step (see Section V: Important Technical Notes, A: Growth and Treatment of Cell Lines). A basic schematic of the steps involved in the G-LISA® is shown in Figure 1. Typical G-LISA® results are shown in Figure 2.

Figure 1: Simple and Quick Protocol

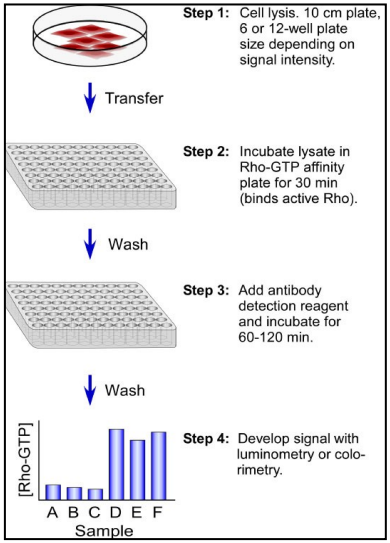
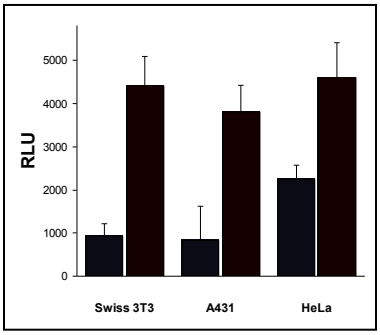


Figure 2: Typical G-LISA® Results



RhoA activation by lysophosphatidic acid (LPA) measured by G-LISA®. Swiss 3T3 (mouse), A431 (human) and HeLa (human) cells were serum starved for 24 hours and treated plus or minus LPA (1 µg/ml for 2 min). 25 µg of cell lysates

Available Assay Formats

G-LISA® assays are available in luminescence and absorbance based formats (see Table 2).

Table 2: Comparison of Luminescence and Absorbance G-LISA® assays

	Luminescence	Absorbance
Assay Time	<3 h	<3 h
Cell material per assay	10-50 µg protein (12-well plate)	10-50 µg protein (12-well plate)
Measurement parameters	High gain, 10-100 ms read per well	490 nm (Cat. # BK124)
*Detection Limit**	0.025 ng RhoA (Cat. # BK121)	0.050 ng RhoA (Cat. # BK124)
Linear Range of assay	0.025 – 1.0 ng	0.05 – 2 ng
cv of eight replicates	16%	12%
High throughput compatible	Yes	Yes

*Detection limit for these assays was determined by titrating the amount of constitutively active recombinant RhoA in the wells.

Limited Use Statement

The G-LISA® kits are based on patented technology developed at Cytoskeleton Inc. (Patent# 7,763,418 B2). 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 96 assays. When properly stored, kit components are guaranteed stable for a minimum of 6 months. You can assay anywhere from 2 to 96 samples at a time for your own convenience. Table 3 summarizes the kit contents.

Table 3: Kit Contents

Reagents	Cat. # or Part # *	Quantity	Storage
96 well Rho-GTP binding plate	Part # GL62	12 strips of 8 wells	Desiccated 4°C
Anti-RhoA antibody	Part # GL01A	2 tubes, lyophilized	Desiccated 4°C
Secondary antibody - horseradish peroxidase conjugate (HRP)	Part # GL02	1 tube, lyophilized	Desiccated 4°C
Rho control protein (constitutively active RhoA)	Part # RHCA Similar to Cat. # R6301	12 tubes, lyophilized	Desiccated 4°C
Cell Lysis Buffer	Part # GL36	1 bottle, lyophilized	Desiccated 4°C
Binding Buffer	Part # GL37	1 bottle, lyophilized	Desiccated 4°C
Wash Buffer	Part # PE38	1 tablet	Room temperature
Antigen Presenting Buffer	Part # GL45	1 bottle, 30 ml	Room temperature
Antibody Dilution Buffer	Part # GL40	1 bottle, lyophilized	Desiccated 4°C
HRP Detection Reagent A	Part # GL41	1 bottle, 3.5 ml	4°C
HRP Detection Reagent B	Part # GL42	1 bottle, 3.5 ml	4°C
Precision Red™ Advanced Protein Assay Reagent	Part # GL50 (available as 500 ml size Cat. # ADV02)	1 bottle, 100 ml	Room temperature
Protease Inhibitor Cocktail	Cat. # PIC02	1 tube, lyophilized	Desiccated 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.**

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

- Cold 4°C PBS buffer (10 mM phosphate buffer pH 7.4, 140 mM NaCl, 3 mM KCl)
- Cell scrapers
- Liquid nitrogen for snap freezing cell lysates.
- Multi-channel or multi-dispensing pipettor for 25-200 µl range.
- Multi-channel pipettor solution basins (available from VWR Cat. # 21007-970). Used for liquid handling.
- Vortex for mixing cell lysate and Binding Buffer solutions.
- Two orbital microplate shakers. Optimal shaker speed is 400 rpm (200 rpm is the minimal speed required). One at room temperature and one at 4°C
- Microplate luminometer (see Section V: Important Technical Notes for information on settings etc.)

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 shown in Table 4:

Table 4: Component Storage and Reconstitution

Kit Component	**Reconstitution**	**Storage Conditions**
96 well Rho-GTP binding plate	It is imperative to keep the plate in the sealed desiccant bag with desiccant at all times. Reconstitution is not necessary prior to the start of the assay. The protective white powder pellet in each well of the plate may become detached from the bottom of the well during shipping. This will not affect the assay performance. Pellets should be tapped to the bottom of the well prior to resuspension.	Store desiccated at 4°C
Anti-RhoA antibody	Centrifuge briefly to collect the pellet in the bottom of the tube. Dissolve the powder in each tube in 20 µl of PBS.	Store at 4°C
Secondary antibody HRP	Centrifuge briefly to collect the pellet in the bottom of the tube. Dissolve the powder in 80 µl of PBS. Do not use sodium azide in combination with this antibody as it will inactivate the HRP.	Store at 4°C
Rho control protein (12 tubes)	Each tube is good for one experiment. Reconstitution is not necessary until starting the assay (see table 5).	Store desiccated at 4°C
Cell Lysis Buffer	Reconstitute in 100 ml of Milli-Q water. This solution may take 5-10 min to resuspend. Use a 10 ml pipette to thoroughly resuspend the buffer.	Store at 4°C
Binding Buffer	Reconstitute in 5 ml of Milli-Q water. This solution may take 5-10 min to resuspend. Use a 5 ml pipette to thoroughly resuspend the buffer.	Store at 4°C
Wash Buffer	Reconstitute in 1 L of Milli-Q water. This solid will take 45-60 min to resuspend. A magnetic stir bar and stir plate can be used to help resuspension.	Store at room temperature
Antigen Presenting Buffer	No reconstitution necessary.	Store at room temperature
Antibody Dilution Buffer	Reconstitute in 15 ml of Milli-Q water.	Store at 4°C
HRP Detection Reagent A	No reconstitution necessary.	Store at 4°C
HRP Detection Reagent B	No reconstitution necessary.	Store at 4°C
Precision Red™ Advanced Protein Assay Reagent	No reconstitution necessary.	Store at room temperature
Protease Inhibitor Cocktail	Reconstitute in 1 ml of dimethyl sulfoxide (DMSO) for 100x stock.	Store at 4°C. The cocktail will freeze at 4°C

A) **Updated Version (3.1) Review**

The following updates from version 2.8 should be noted.

1. The RhoA antibody has been updated from 1 tube of 40 µl to 2 tubes of 20 µl each. This was done to assist customers in preventing contamination of antibody when performing multiple experiments and to allow for longer storage.
2. Kit replaces GL38 Wash Buffer with Bottle with PE38 Wash Buffer-tablet only. The tablet composition is identical, only the bottle was removed. This was done for space considerations only and has no effect on kit functionality.
3. The manual has been reformatted to fit a 5.5 x 8.5 page format.

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.

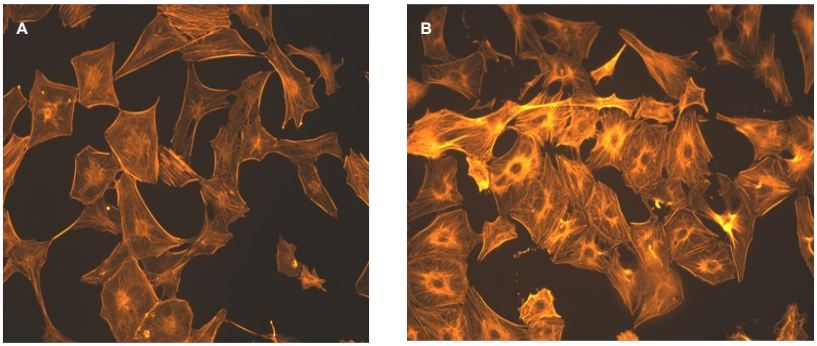
Adherent fibroblast cells such as 3T3 cells should be ready at 50-70% confluency or for non-adherent cells, at approximately 3 x 10^5 cells per ml.

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. A list of known Rho 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 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. Figure 3 below shows rhodamine phalloidin stained Swiss 3T3 cells that have been serum starved (A) and calpeptin (Cat. # CN01) stimulated (B). Rho activation by calpeptin causes the formation of characteristic stress fibers.

Figure 3: Rhodamine Phalloidin Stained Serum-Starved and Calpeptin-Treated Cells



Swiss 3T3 cells were grown at 37°C, 5% CO_2 and 95% humidity in 100 mm dishes containing a small glass coverslip. Cells were grown in Dulbecco's modified Eagle's medium plus 10% calf bovine serum to 60% confluency. For serum starvation, media was changed to 0.5% calf bovine serum for 24 h then to 0% calf bovine serum for a further 24 h. After this time, one dish was treated with calpeptin (Cat. # CN01; 0.1 mg/ml final) for 30 min and the other dish treated with carrier only (DMSO). After incubation, the coverslips were removed from the dishes and stained with rhodamine phalloidin (A, serum starved cells; B, calpeptin treated cells). The remaining cells were lysed and processed in a G-LISA® assay. G-LISA® results showed calpeptin treated cells expressed 2 fold the Rho activity of the serum starved cells (data not shown).

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) **Assay Preparation For G-LISA®**

It is critical to get the assay components ready before preparing cell lysates or thawing previously prepared lysates because the GTP-bound form of Rho is very labile and should be assayed as soon as possible after cell lysate preparation or thawing frozen lysates.

Table 5: Assay Preparation for G-LISA®

Reagent	Preparation
Rho-GTP binding 96 well plate	1) Remove plate from 4°C and keep in its protective bag. Place on your bench at room temperature for 30 min. 2) Do not remove the plate (or strips) from the bag until immediately prior to the experiment.
Milli-Q water	30 ml placed on ice.
Binding buffer	Embed bottle in ice.
Protease Inhibitor Cocktail	Resuspend in 1 ml of dimethyl sulfoxide (DMSO) and keep at room temperature.
Lysis Buffer	1) Determine volume of Lysis Buffer needed per culture vessel by looking at Table 6. 2) Determine **total volume** of Lysis Buffer needed by multiplying the lysis volume per culture vessel (µl) by number of vessels x 1.3. 3) Aliquot this volume of Lysis Buffer into a clearly labeled tube and place in ice. 4) Add 10 µl of protease inhibitor cocktail per ml of aliquoted Lysis Buffer. 5) Mix well and leave on ice. 6) Lysis Buffer needs to be **ice cold**.
Rho control protein	Dissolve one tube in 500 µl Lysis Buffer and leave on ice. This solution is good on ice for up to 8h.
PBS pH 7.2	Phosphate-buffered saline is not provided in the kit. It should be prepared prior to the assay and placed on ice for at least 30 min to ensure that it is ice cold.
Anti-RhoA antibody	Have primary antibody stock ready on ice. For each 8-well strip, you will need to mix 2 µl antibody with 500 µl Antibody Dilution Buffer. This dilution step should be performed just prior to use as detailed in assay protocol.
Secondary antibody	Have secondary antibody stock ready on ice. For each 8-well strip, you will need to mix 2 µl antibody with 500 µl Antibody Dilution Buffer. This dilution step should be performed just prior to use as detailed in assay protocol.

Preparation for G-LISA® (Continued)

Reagent	Preparation
Antibody Dilution Buffer	Place reconstituted buffer on the bench and use at room temperature.
Wash Buffer	Place on the bench and use at room temperature.
Antigen Presenting Buffer	Place on the bench and use at room temperature.
HRP Detection Reagents A and B	This can be removed from 4°C and placed on your bench at room temperature. For each 8-well strip, mix 250 µl of each reagent in a new tube. Make sure to use separate pipette tips for dispensing each solution.
Precision Red™ Advanced Protein Assay Reagent	Place on the bench and use at room temperature.
Sample Tubes	Label 1.5 ml microfuge tubes, three for each sample expected. Two tubes are needed per sample as there is a clarification step that requires removal of the supernatant to a fresh tube.

D) **Timing and Intensity of Rho 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. Examples of known Rho activators are given in Appendix 2. For potent activators such as calpeptin or LPA, the intensity of maximal Rho 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 and the signal will be more like 2 fold activation. 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, which fit nicely into a 6 well culture plate (The time course is also recommended for Rho inactivation studies).

In practical terms the timed experiment must be performed in reverse order, that is, starting with the 30 min time point, then waiting 18 min to initiate the 12 min time point. Likewise, a further 6 min to initiate the 6 min time point, and so on down to the zero time point where you process cells on ice and immediately wash with ice cold PBS as outlined in the Assay Protocol. Accurate and quick addition of the ice cold PBS is critical for quenching the time points.

E) **Rapid processing of cells**

GTP bound (active) Rho is a labile entity and the bound GTP is susceptible to hydrolysis 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

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 6 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 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 (<0.4 or >2.0 mg/ml), it is recommended to adjust the amount of Lysis Buffer depending on your cell type and plate type. Table 6 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.

Table 6: 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	70
60 mm dish	21	3.0	100
100 mm dish	56	10.0	250
150 mm dish	148	15.0	700
6-well cluster plate	9.5 / well	3.0	70
12-well cluster plate	4 / well	1.5	35
T-25 Flask	25	4.0	100
T-75 Flask	75	10.0	500
T-150 Flask	150	15.0	700

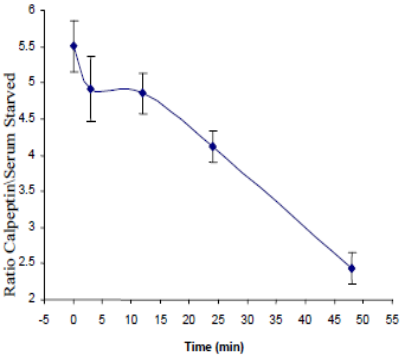
The time period between cell lysis and addition of lysates to the wells is critically important (see Figure 4). Take the following precautions:

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. Preferably, use lysates immediately, do not leave on ice for longer than 10 min. As an alternative, particularly in instances where large numbers of samples are being processed, cell lysates may be immediately frozen for use at a later time, however, lysates **must** be snap frozen in liquid nitrogen and stored at -70°C. Lysates should be stored at -70°C for no longer than 14 days and thawing should be in a room temperature water bath, followed by rapid transfer to ice and immediate use in the assay.

Figure 4: Loss of Rho Activity Signal Over Time



Serum-starved and calpeptin-treated cells were lysed as described in the Assay Protocol. The ratio of activated Rho in calpeptin versus serum starved cells was quantitated using the G-LISA® assay at various time-points post cell lysis. After approximately 45 minutes on ice, the ratio of active RhoA in calpeptin versus serum starved cells was approximately half that of the cells lysed at time zero. It should be noted that the reduced values for the ratio of active RhoA between calpeptin and serum starved cell lysates is a result of a combination of decreasing calpeptin values and increasing serum starved values.

F) Protein Concentration Equivalence

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 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. We highly recommend that the final concentration of equalized lysates lies between 0.4–2 mg/ml. Protein concentrations below 0.4 mg/ml often will not work with sufficient accuracy to warrant performing the assay. It is not necessary to equalize protein concentrations if the variation between them is less than 10%. The equalized cell extracts should be used for G-LISA™ Rho assay immediately.

The Precision Red™ Advanced Protein Assay Reagent (Part # GL50) is included with the kit in order to measure protein concentration with a rapid one-step procedure. Precision Red™ Advanced Protein Assay Reagent is ideal for this analysis because it is detergent compatible, rapid, and simple to perform. The protein assay can be performed in a 1 ml cuvette format as described in the Assay Protocol section. Alternatively, a 96-well format can be used where 10 µl of sample is pipetted into a well followed by the addition of 300 µl of Protein Assay Reagent. In this case the absorbance reading at 600 nm is multiplied by 3.75 to obtain the protein concentration in mg/ml.

G) Assay Linearity

The assay is linear between to 1 ng of bound activated RhoA. The positive control protein is at 1 ng, therefore any assay readings that are greater than the positive control will be out of the linear range of the assay. In such cases you should reduce the amount of total cell protein per assay. The lower level of detection of the assay is approximately 20% above the background reading of Lysis Buffer only.

H) **Use of a Multi-channel Pipettor**

When processing more than 16 wells, it is imperative to use a multi-channel or multi-dispensing pipettor with a range of 25 to 200 µl per dispense. Critical steps such as lysate addition, post-binding wash step and the Antigen Presenting Buffer step all have requirements for accurate and timely additions. For example, the Antigen Presenting Buffer must not be left in the wells longer than 2 min because significant signal can be lost here, a 10 min delay here will cause a loss of 70% of your signal. Attempting to perform >16 assays with a single channel pipettor will also increase the likelihood of allowing wells to dry out before reagent addition can be completed, resulting in variable signals. Therefore, use a multi-channel or at least a multi-dispensing pipettor wherever possible. If neither of these pipettor options is available, we highly recommend that you limit each experiment to a maximum of 16 wells.

I) **Removal of Solutions from Wells**

Removal of solutions from the wells is accomplished by turning the plate upside down and flicking out the well contents into a waste bin. This is followed by patting the plate several times on a paper towel to get rid of residual solution. It has been found that the complete removal of solutions from the well requires a vigorous flick of the plate and a vigorous series of pats onto paper towels (5-7 hard pats). The complete removal of solution from wells between steps of the G-LISA® is very important as it avoids high background readings in the buffer only wells. As absolute values differ markedly between different luminometers, a guide for acceptable backgrounds is that the background reading should be 6-9 times lower than the positive control in any given luminometer.

J) **Plate Shaker Recommendations**

It is recommended to use an orbital plate shaker at 400 rpm. As a back-up you can use a 200 rpm orbital shaking culture incubator or a normal orbital rotating platform. Signals will be lower with the 200 rpm option.

K) **Luminometer Settings**

Luminometers vary widely in their sensitivity and absolution readings. It is therefore recommended to run a G-LISA® assay with blank and positive control to determine that you are in the linear range of the assay. When in the linear range of the assay, the positive control should read 6-9 fold higher than the blank wells. Table 7 gives guidelines for luminometer settings:

Table 7. Luminometer settings

Parameters	Description and Recommendations
Gain	Gain controls the sensitivity of the machine. Most luminometers do not allow manual alteration of gain and use an auto-calibration or limited calibration function. It is important to contact the luminometer manufacturer or consult the user's manual to determine the best way to alter the machine sensitivity. If gain can be altered one should read at low, medium, and high gains to determine the reading within the linear range of the assay (positive control should be 6-9X higher than the blank). Gain range varies with instrument, for example gain in the Tecan GmbH SpectroFluor Plus ranges from 0—150 (where 150 is the highest).
Integration Time	This parameter can be varied on most machines. It is a good idea to set the machine as the lowest integration time (usually 10-100 ms). Integration times greater than 200 ms are likely to read out of the linear range of the assay and may require lowering of gain or dilution or primary and/or secondary antibodies (see below)
Shaking	Most machines give the shaking option. The recommended setting is 5 s shake, medium orbital speed before read. This option is not essential to the assay.
Temperature	Room temperature
Plate Type	Any setting that specifies 96 well flat, white will be sufficient
Filters	Luminescence does not require excitation or emission filters. The filter spaces should be left blank. If this is not an option, excitation can be set at any value and emission should be set between 400-500 nm, with 430-445 nm as optimal setting.

L) **Assay Optimization of Antibody Concentrations**

If the G-LISA® readings are not in the linear range even when your luminometer is set at lowest sensitivity and integration time then it will be necessary to vary the antibody dilutions. Furthermore, if the blank reading is greater than 9-10 million when using the 1/250 primary and secondary antibody dilutions and the luminometer is set at lowest sensitivity, it is recommended to go to higher antibody dilutions. The following dilutions are recommended:

Primary	Secondary	Condition	Method
1:250	1:250	These are the current recommended conditions for the assay	Each 8 well strip required 500 µl of reagent. 1. **Primary Ab**: 2 µl of primary antibody to 500 µl of antibody dilution buffer. 2. **Secondary Ab**: 2 µl secondary antibody to 500 µl of antibody dilution buffer.
1:500	1:1000	2 fold increased dilution of primary Ab and 4 fold increased dilution of secondary Ab.	Each 8 well strip required 500 µl of reagent. For accuracy of pipetting we recommend the following antibody dilution method. 1. **Primary Ab**: 2 µl of primary antibody to 1000 µl of antibody dilution buffer. 2. **Secondary Ab**: a. 2 µl secondary antibody to 8 µl of phosphate buffered saline (PBS) pH 7.4. b. Add 5 µl of dilution secondary antibody to 500 µl of antibody dilution buffer.
1:500	1:2000	2 fold increased dilution of primary Ab and 8 fold increased dilution of secondary Ab.	Each 8 well strip required 500 µl of reagent. For accuracy of pipetting we recommend the following antibody dilution method. 1. **Primary Ab**: 2 µl of primary antibody to 1000 µl of antibody dilution buffer. 2. **Secondary Ab**: a. 2 µl secondary antibody to 18 µl of phosphate buffered saline (PBS) pH 7.4. b. Add 5 µl of dilution secondary antibody to 500 µl of antibody dilution buffer.

Please inquire to Technical assistance for help is setting up other machines (call 303-322-2254 for immediate help, or e-mail tservice@cytoskeleton.com for assistance within 24 h).

VI: Assay Protocol

It is crucial to the success of this assay that the section entitled "Important Technical Notes" be read thoroughly and followed accurately. The ⚠ sign indicates steps that have particularly critical "Important Technical Notes". Have copies of Appendices 3 and 4 ready to fill out as you go through the assay. Filling these out will be a good reference for you and of vital importance in case you need technical support.

⚠ STEP 1: Assay Preparation

At least one hour prior to beginning the assay, prepare all G-LISA® assay components as described in Section IV and Section V: Important Technical Notes, Table 5. Use the check-off list below to confirm that the following reagents are ready:

- Rho-GTP plate, at room temperature in the desiccant bag □
- Wash Buffer, resuspended at room temperature □
- Precision Red™ Advanced Protein Assay Reagent, room temperature □
- Distilled water, 30 ml, ice cold □
- Binding Buffer, ice cold □
- Lysis Buffer, ice cold with protease inhibitors (see Table 5) □
- PBS, ice cold (see Table 5) □
- 1.5 ml microfuge tubes, ice cold □
- RhoA Control Protein, resuspended in Lysis Buffer (500 µl per tube) on ice □
- Antibody Dilution Buffer, room temperature □
- Binding Buffer, ice cold □

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. Save a small amount of lysate before you snap freeze them for protein quantitation.

Cells Grown in Tissue Culture Vessels as Monolayers

1. Treat cells with Rho activator (or inactivator) as your experiment requires.
2. After treatment, place culture vessel on ice, aspirate media, wash with ice cold PBS. See Table 6, Section V for recommended volumes.
3. 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.
3. Lyse cells in an appropriate volume of ice-cold Cell Lysis Buffer. See Table 6, Section V for recommended volumes.
4. 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.
5. Transfer lysates into the pre-labeled sample tubes on ice.
6. Immediately clarify by centrifugation at 10,000 x g, 4°C for 2 min. (For high throughput screens or well characterized experiments, the clarification step can be omitted).
7. At this point each lysate volume should not exceed 130% of the original Lysis Buffer volume.
8. Save at least 20 µl of lysate aside in a microcentrifuge tube for protein quantitation assay.
9. Aliquot and snap freeze the remaining cell lysates in liquid nitrogen. Keep them at **-70°C** for future use. It is recommended to aliquot into 100 µl volume size per condition (sufficient for triplicate G-LISA® reactions).
10. Measure lysate protein concentrations.
    A. Add 20 µl of each lysate or Lysis Buffer into disposable 1 ml cuvettes.
    B. Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Part # GL50) to each cuvette.
    C. Incubate for 1 min at room temperature.
    D. Blank spectrophotometer with the Lysis Buffer + Protein Assay Reagent at 600 nm.
    E. Read absorbance of lysates samples.
    F. Multiply the absorbance by 5 to obtain the protein concentration in mg/ml.

12. Move on to next time point or condition and process the lysate as described above.

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 0.3 mg/ml. Specifically, we recommend a final concentration of 0.5 mg/mL for 3T3 cells and 1 mg/ml for HeLa cells. 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:

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

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 G-LISA® assays simpler. Be aware of the length of time cell lysates stay on ice (do not exceed 10 min or Rho GTP hydrolysis will occur).

STEP 3: G-LISA® Assay

Before beginning the assay it is highly recommended to calibrate your luminometer to read within the linear range of the assay. The assay is in the linear range when the positive control reads 6-9X higher than the buffer blank. Luminometer calibration is described in Section V, Sub-section (K) of this manual.

1. Aliquot 60 µl of Lysis Buffer into a labeled microfuge tube and dilute with 60 µl of ice cold Binding Buffer. Keep on ice. This is your buffer blank control.

2. Aliquot 12 µl of Rho Control Protein into a labeled microfuge tube and dilute with 48 µl of Cell Lysis Buffer and 60 µl of Binding Buffer. Vortex for 3-5 s on high and place on ice. This is your positive control sample. NOTE: The unused RhoA control protein must be discarded as it will denature.

NOTE: If you have not yet calibrated the luminometer as outline in Section V Sub-section K (Luminometer Settings), you should consider running the blank (Lysis Buffer only) and Rho Control Protein only in this assay. This will allow you to calibrate the luminometer and assess appropriate antibody dilutions before running your samples.

3. Take the Rho affinity plate out of its bag. Gently peel up the seal from the strips and pull out the number of strips required. Place strips in the extra strip holder provided, and place on ice. Immediately after removing the strips, place the rest of the plate back in the pouch with desiccant and place back in storage.

4. Dissolve the powder in the wells with 100 µl ice cold water

5. Thaw the snap frozen cell lysates in a ROOM TEMPERATURE water bath. Immediately place on ice after they are thawed.

6. Based on the calculation of equalization, add required amount of ice cold Lysis Buffer to respective tubes to equalize all lysate concentration.

   Note: It is recommended to calculate the dilution factors required BEFORE thawing out lysates as this allows rapid sample processing.

7. Immediately aliquot sufficient lysate for duplicate (60 µl) or triplicate (90 µl) assays into fresh ice cold microcentrifuge tubes.

8. Add an equal volume of ice-cold Binding Buffer to each tube and mix well with a pipette . Keep on ice.

9. Completely remove the water from the microplate wells as follows:

   Complete removal of solutions from the well requires a vigorous flick of the plate and a vigorous series of pats onto paper towels (5-7 hard pats). The complete removal of solution from wells between steps of the G-LISA® is very important as it avoids high background readings in the buffer only wells. As absolute values differ markedly between different luminometers, a guide for acceptable backgrounds is that the background reading should be 5-7 times lower than the positive control reading in any given luminometer.

10. Put plate back on ice.

11. Immediately add 50 µl of equalized cell lysate to duplicate or triplicate wells.

12. Pipette 50 µl of buffer blank control into duplicate wells.

13. Pipette 50 µl of RhoA positive control into duplicate wells.

14. Immediately place the plate on a cold orbital microplate shaker (400 rpm recommended, 200 rpm minimum) at 4°C for exactly 30 min.

NOTE: An ORBITAL microplate shaker set to a minimum of 200 rpm must be used. Slower shakers or rockers will not be sufficient.

15. During the incubation, dilute the anti-RhoA primary antibody to 1/250 in Antibody Dilution Buffer by adding 2 µl of antibody to every 500 µl Antibody Dilution Buffer. Note: The final volume of 500 µl is adequate for one strip (8 wells).

NOTE: If the assay is determined to be out of the linear range of the luminometer (positive control should be 6-9X higher than buffer only reading) or the blank reading is greater than 9-10 million when the luminometer is set at the least sensitive level then further antibody dilutions are recommended. See Table 8, Section V, Sub-section L in this manual.

16. After 30 min, flick out the solution from the wells and wash twice with 200 µl Wash Buffer at room temperature using a multi-channel pipettor. Do not leave this plate unattended at this time. Vigorously remove the Wash Buffer after each wash by flicking and patting the plate as detailed in step 9.

17. Place the plate on the bench at room temperature.

18. **Immediately pipette 200 µl of room temperature Antigen Presenting Buffer into each well using a multi-channel pipettor and incubate at room temperature for exactly 2 min.**

19. Vigorously flick out the Antigen Presenting Buffer, patting the inverted plate 5-7 times on a stack of paper towels as outlined in step 9.

20. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer. Vigorously remove Wash Buffer after each wash as detailed in step 9.

21. Add 50 µl of diluted anti-RhoA primary antibody to each well and leave the plate on the orbital microplate shaker (200-400 rpm) at room temperature for 45 min.

22. During the primary antibody incubation, dilute the secondary HRP labeled antibody to 1/250 in Antibody Dilution Buffer by adding 2 µl of antibody to every 500 µl Antibody Dilution Buffer. Note: The final volume of 500 µl is adequate for one strip (8 wells).

NOTE: If the assay is determined to be out of the linear range of the luminometer (positive control should be 6-9X higher than buffer only readings) then further antibody dilutions are recommended, for details see Table 8, Section V, Sub-section L in this manual.

23. Vigorously flick out the anti-RhoA primary antibody, patting the inverted plate 5-7 times on a paper towel as outlined in step 9.

24. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer. Vigorously remove Wash Buffer after each wash as detailed in step 9.

25. Add 50 µl of diluted Secondary antibody to each well and leave the plate on a microplate shaker (200–400 rpm) at room temperature for 45 min.

26. During the secondary antibody incubation prepare the HRP detection reagent. The HRP reagent is in two parts (A & B) which should mixed in equal volumes prior to adding 50 µl to each well. For each strip of 8 wells, mix 250 µl Reagent A with 250 µl of Reagent B into a new tube. The mixture is stable at room temperature for 1h.

27. Vigorously flick out the secondary antibody, patting the inverted plate 5-7 times on a paper towel as outlined in step 9.

28. Wash the wells three times with 200 µl of room temperature Wash Buffer, Vigorously remove Wash Buffer after each 200 µl wash as detailed in step 9.

29. Pipette 50 µl of the mixed HRP detection reagent into each well detect the luminescence signal using a microplate luminescence reader as described in Section V: Important Technical Notes. Readings must be taken within 3-5 min to obtain maximum signal. Designate Lysis Buffer only wells as the assay blank.

VII: Data Analysis

1. It is recommended to use the Lysis Buffer wells as reference blanks in all studies with this kit. Based on the operator designating the appropriate wells, most machines have associated protocols that perform this operation automatically. Call Technical Help for the company supplying the plate reader for information on how to perform this function. When the data are “Lysis Buffer subtracted” (Lysis Buffer only samples have been allocated as Blanks in the assay), then you can import them into a simple graph software like Excel or Sigma Plot. Alternatively, the Lysis Buffer background can be subtracted manually or in the spreadsheet application.

2. Data should be arranged in columns where the headings are “Sample”, “Mean”, “Standard Deviation”, “rep1”, “rep2”, “rep3” and “rep4” for the number of replicates performed on each sample. E-mail tservice@cytoskeleton.com or visit www.cytoskeleton.com for a free Excel Template.

3. List your samples under the “Sample” column in the same order that they were assayed in the plate.

4. Enter the following formula into the first sector under “Mean”, “=average(Xn:Yn)” where X = the column designator for “rep1”, Y = column designator for “rep4”, and n= row designator of the row that you are working on. Repeat for each sector under the “Mean” header until there are sufficient rows to cover the number of samples in your experiment.

5. Enter the following formula into the first sector under “Standard Deviation”, “=stdev (Xn:Yn)” where X = the column designator for “rep1”, Y = column designator for “rep4”, and n= row designator of the row that you are working on. Repeat for each sector under the “Standard Deviation” header until there are sufficient rows to cover the number of samples in your experiment.

6. Enter your replicate data into rep1, rep2 etc. It doesn’t matter if you only have duplicates because the program will ignore any sectors that do not contain data. The program will calculate the Mean and Standard Deviation of your replicates.

7. When the data has been entered select the Sample, Mean and Standard Deviation data sectors by the click and drag method. Then select the chart making function, in Excel this looks like a clickable square with a mini-bar chart inside. This will guide you through the chart making process with the data you have selected. Choose “column chart” initially, designate the Mean numbers for input values. The Standard Deviation column for the y-axis error bars needs to be designated after the Mean numbers chart is made. This is achieved by double clicking on the graph bars, and selecting the “Y-axis error” tab, then entering the location of the Standard Deviation data by clicking the “Custom” option and selecting the area in the worksheet. E-mail tservice@cytoskeleton.com for a free Excel Template. An example of a typical Excel layout and data plot is shown in Figures 6A and B.

Figure 5A: Typical Excel Layout

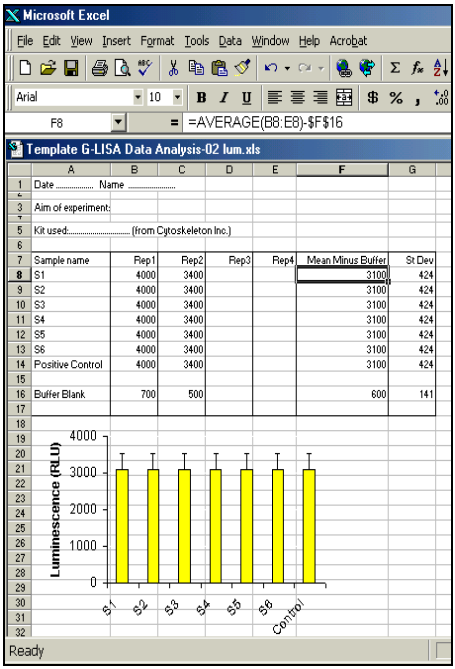
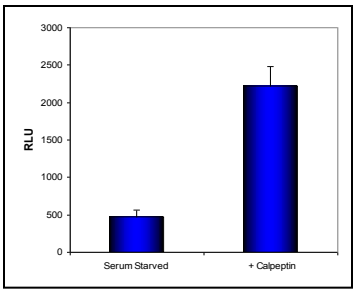


Figure 5B: Typical G-LISA® Results



RhoA activation by calpeptin measured by G-LISA®. Swiss 3T3 (mouse) cells were serum starved for 24 hours and treated plus or minus calpeptin (100 µg/ml for 30 min). 25 µg of cell lysates were subjected to the BK121 G-LISA® assay. Data was read at 150 gain, 10 ms integration time on a Tecan GmbH SpectroFluor Plus.

VIII: Troubleshooting Guide

Observation	Possible cause	Remedy
Weak signal or no signal in all wells	1. Plate reader setting is not optimal. 2. Concentration of antibodies is too low. 3. The wells were allowed to dry out during the experiment. 4. The plate was allowed to get damp during storage. Well contents will appear sticky and opaque. 5. A step or component of the assay was omitted.	1. Optimize luminometer as described in Section V Sub-section L. 2. Follow the recommendation of dilution of antibody in the manual. 3. Do not remove the solution in the wells unless the solution of next step is ready. 4. Store the plate in the desiccant bag with the bag securely sealed. Keep the cover on the plate. 5. Read instructions carefully.
High signal in all wells	1. Luminometer settings are too high. 2. Concentration of antibodies are too high. 3. Insufficient washes were performed	1. Reduce gain and/or integration time on luminometer and read again. 2. Follow the recommended dilution of antibodies in the manual. If still too high, an antibody titration is necessary to optimize your results (See Section V Sub-section L). 3. Follow the instructions for the washing in the manual.
Background readings are high (background should be 1/6-1/9 of positive control reading)	1. Inefficient removal of solutions from G-LISA® wells. 2, Antibody concentrations are too high for sensitivity of luminometer.	1. Background should be approximately 1/6th to 1/9th of the positive control reading. Complete removal of solutions from the wells is required to product a clean assay. Vigorous flicking and patting of the inverted plate is required to completely remove solutions from the wells after each step is complete. See Important Technical Notes and the G-LISA® method for details. 2. In some cases where very sensitive luminometers are used, it will be necessary to dilute primary and/or secondary antibodies to obtain a reading within the linear range of the assay.
Induced sample does not give significant signal increase	1. Poor inducer activity 2. Technique not rapid or cold enough 3. Too much extract in the wells or the concentration of extract is too high. 4. The endogenous GTP-RhoA level is too high. 5. Tissue culture cells not correctly serum starved. 6. Temperature of lysis and incubation is not 4°C. 7. The Binding buffer is not pre-cooled at 4°C.	1. Purchase a fresh vial of inducer. 2. Read instructions carefully and compare with your Experiment Record Sheet 3. The linear range of the assay is 0.02 – 1 ng RhoA. Generally 10-50 µg of total cell extract falls within the linear range. 4. Titrate down the amount of extract to be added. 5. Details of how to serum starve cells is given in Appendix 1. It is a good idea to stain cells with rhodamine-phalloidin to qualitatively determine success of serum starvation and induction. 6. Lyse cells on ice and keep all cell lysis and binding buffers on ice. 7. Make sure the buffer was stored in the fridge and kept on ice before use.

Observation	Possible cause	Remedy
Significant variation between duplicate/ triplicate samples.	1. Incorrect volume of solutions for each step added in the wells. 2. Inaccurate pipetting. 3. Bubbles in wells.	1. Follow the instruction for recommended volume in the manual. 2. A multi-channel pipettor is recommended. 3. Remove bubbles.
Positive control not working	1. Positive control protein was stored after reconstitution.	1. Use a fresh tube each time, there are 12 per kit.

IX: References

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X: G-LISA® Citations

Rho G-LISA®

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Section XI: Related Products

ITEM CAT. #	Description	GTPase	Quantity
BK124*	G-LISA® for RhoA Activation Assay (Absorbance based)	RhoA	96 assays
BK125*	G-LISA®for Rac Activation Assay (Absorbance based)	Rac	96 assays
BK126*	G-LISA® for Rac1 Activation Assay (Luminescence based)	Rac1	96 assays
BK127*	G-LISA® for Cdc42 Activation Assay (Absorbance based)	Cdc42	96 assays
BK128*	G-LISA® for Rac1 Activation Assay (Absorbance based)	Rac1	96 assays
BK129	G-LISA® for Ral Activation Assay (Absorbance based)	Ral	96 assays
BK150	Total RhoA ELISA	RhoA	96 assays
BK008	Ras Activation Assay Biochem Kit™(Pull down assay)	Ras	25 assays
BK034	Cdc42 Activation Assay Biochem™ Kit (Pull down assay)	Cdc42	25 assays
BK035	Rac Activation Assay Biochem Kit™ (Pull down assay)	Rac	25 assays
BK036	RhoA Activation Assay Biochem Kit™(Pull down assay)	RhoA	25 assays
BK100	RhoGEF exchange assay(Fluorimetric)	Rho/Ras	30-100 assays
BK105	RhoGAP assay(Colorimetric)	Rho/Ras	30-100 assays
BK005	Actin Staining Biochem Kit™ *(for rhodamine- phalloidin staining of actin filaments in fixed cells)*	N/A	300 – 1000 assays
ARC03	Anti-Rac1 mouse monoclonal antibody	Rac1	20 assays
CT04	Cell permeable Rho inhibitor	Rho	1 x 25 µg 5 x 25 µg 20 x 25 µg
RT02-A RT02-B	Rhotekin-RBD beads	Rho effector	2 x 2 mg 6 x 2 mg
PAK02-A PAK02-B	PAK-PBD beads	Rac/ Cdc42 effector	1 x 500 µg 4 x 500 µg
*Patent Number 7,764,418 B2			

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

Reagents needed

- Control state cells and Responsive state cells (e.g. serum-starved cells and LPA-treated cells).
- PBS solution (10 mM phosphate buffer pH 7.2, 140 mM NaCl, 3 mM KCl)
- Rhodamine Phalloidin stock (14 μM in methanol, Cat. # PHDR1)
- Fixative Solution (4% formaldehyde in PBS)
- Permeabilization Buffer (0.5% Triton in PBS)
- Antifade Mounting Medium

All above reagents (except cells) are available in a convenient kit format from Cytoskeleton Inc. (Actin Staining Biochem Kit™, Cat. # BK005)

Method

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

1. Cells are seeded at a density of 3–5 x 10^5 cells on a 10 cm diameter plate containing two 13 mm diameter glass coverslips.
2. Once cells reach 50-60% confluency they are washed once in serum free medium and then incubated in fresh medium containing 0.5% serum for 24 h. After this time, cells are changed into fresh serum free medium for 16-24 h to obtain serum-starved cultures.
3. After serum starvation, one coverslip is processed for actin staining as described below in “Actin Staining”.
4. LPA (or other Rho activator) is added to the remaining cells to 1 μg/ml final concentration and the second coverslip is removed after 5 min.
5. The coverslip is processed for actin staining as described below.

Actin Staining

1. Remove coverslip from growth medium.
2. Wash cells once with PBS and incubate in Fixative solution for 10 min.
3. Prepare a 100 nM working stock of rhodamine-phalloidin by diluting 3.5 μl of stock rhodamine-phalloidin (Cat. # PHDR1) into 500 μl of PBS. Keep at room temperature in the dark.
4. Wash once with PBS for 30 s at room temperature.
5. Permeabilize cells by incubating in permeabilization buffer for 5 min at room temperature.
6. Wash once in PBS for 30 s at room temperature.

1. Incubate with working stock rhodamine-phalloidin for 30 min at room temperature in the dark.
2. Wash three times with PBS.
3. Mount cells in mounting medium and allow to set for 60 min in the dark.
4. View actin filaments by fluorescence microscopy (excitation filter 535 nm, emission filter 585 nm).
5. Examples of serum-starved and LPA-treated cells are shown in Figure. 1.

Figure 1. Rhodamine Phalloidin Staining of the Actin Cytoskeleton in Serum-Starved and LPA Treated Cells

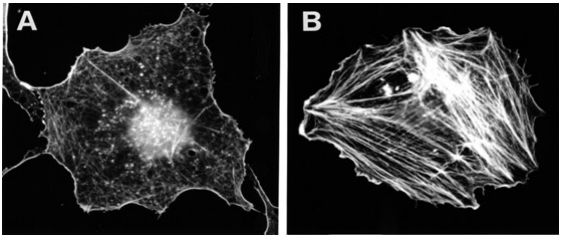

Figure 1. Swiss 3T3 cells serum starved with a two stage step down over two days, 10% → 0.5% → 0.0% serum, prior to actin filament staining with rhodamine-phalloidin. B) Cells treated for 5 min with 1 µg/ml LPA after serum starvation and subsequently stained with rhodamine- phalloidin.

Appendix 2: Known RhoA Activators

Activator*	Treatment	Cell line used	Response	Type of Assay Used	Ref.
Bombesin	10 nM	Swiss 3T3 cells	Maximal activation after 1 min which is sustained for at least 30 min	Actin morphology	2
Calpeptin (protease inhibitor, protein tyrosine phophatase inhibitor)	100 µg/ml	REF-52 fibroblasts & Swiss 3T3 cells adherent	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	9
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	8
Cytochalasin D *(actin filament destabilizer)*	0.5 µg/ml	Swiss 3T3 cells, adherent or suspension	Maximal activation of 2-3 fold after 60 min	Rhotekin-RBD pulldown	8
Fibronectin *(extracellular matrix protein)*	Culture plate is coated with fibronectin	Swiss 3T3 cells	Biphasic regulation after plating cells on fibronectin coated plates. Initial period of low RhoA activity (10-20 min) followed by a 2-7 fold activation peaking at 60-90 min and then dropping to basal levels after 6 h.	Rhotekin-RBD pulldown	8
Lysophospatidic acid (LPA) *(serum lipid & G-protein 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	8
Lysophospatidic acid (LPA)	1 µM	N1E-115 neuronal cells	Maximal activation of 3-5 fold after 3 min	Rho-kinase pull down assay	10
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	11
Serum	5 - 10%	Swiss 3T3 cells, adherent or suspension	Maximal activation of 2-6 fold (10%) & 2-3 fold (5%) after 1-5 min	Rhotekin-RBD pulldown	8
Sphingosine-1-phosphate (serum lipid & G-protein coupled receptor agonist)	1 µg/ml	Swiss 3T3 cells, adherent or suspension	Maximal activation of 2-3 fold after 2 min for 3T3 cells and 20 min for HUVEC cells	Rhotekin-RBD pulldown	8, 12
Thrombin *(protease, G-protein coupled receptor agonist)*	10 nM	HUVEC human venous endothelial primary cells (fifth passage cells)	Maximal activation of 14 fold after 2 min, dropping to basal after 30 min	Rhotekin-RBD pulldown	12
Vinblastine *(microtubule destabilizer)*	50 µM	MG63 human osteosarcoma cells	Maximal activation after 30 min	Actin morphology	11

Appendix 3: Experiment Record Sheet

Scientist Name …….

Contact Tel. # …….

e-mail …….

Kit Cat. # / Lot # …….

STEP		Comments or Changes
1	Type of cells or tissue ……..	
2	How were the cells prepared prior to lysis?	……. days in culture
		……..% confluency
		…… inducer
		……..mg/ml of protein in lysate
3	How long were the ice cold solutions on ice before lysis?	…….. min
4	Time that cultures were removed from incubator?	…… am or pm
5	Was Binding Buffer added?	…….. Y or N
6	Time that binding reactions were placed on the shaker?	…… am or pm
7	Did you add 50 µl of extract per well?	……. Y or N
8	What locations are the 50 µl Lysis Buffer controls?	……. Wells
9	What speed and time was the shaking for the binding reaction?	…… rpm ……min
10	How long did you wait after the post-binding wash step?	…… s or min
11	What time was the anti-Rho primary antibody reaction started?	……..am or pm
12	What time was the Secondary antibody reaction started?.	..... am or pm
13	What was the time when detection reagent was added?	……..… am or pm
14	What was the time when the plate was read?	…….. am or pm

Technical Assistance: call either 303-322-2254 or e-mail tservice@cytoskeleton.com.

Appendix 4: Plate Record Template

Name of experiment: ……..

Date of experiment: ……

Technical Assistance: call either 303-322-2254 or e-mail tservice@cytoskeleton.com

H	G	F	E	D	C	B	A	
								1
								2
								3
								4
								5
								6
								7
								8
								9
								10
								11
								12

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