RHOA / RAC1 / CDC42 G-LISA GTPASE ACTIVATION ASSAY BUNDLE 3 KITS (24 ASSAYS PER KIT, ABSORBANCE-BASED)

MANUAL COVERSHEET

CATALOG # BK135

PART/CATALOG #	DESCRIPTION
BK124-S	RHOA GLISA ACTIVATION ASSAY KIT
BK127-S	CDC42 GLISA ACTIVATION ASSAY KIT
BK182-S	RAC1 GLISA ACTIVATION ASSAY KIT

V 10.0

G-LISA® Rac1 Activation Assay Biochem Kit™: 24 Assays

(Absorbance Based)

Cat. # BK128-S

UPDATED FORMAT

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com

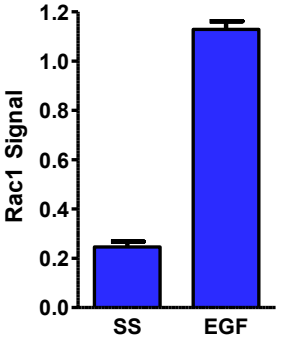
Manual Contents

Assay Principle

The Rac1 G-LISA® kit contains a Rac-GTP-binding protein linked to the wells of a 96 well plate. Active, GTP-bound Rac1 in cell/tissue lysates will bind to the wells while inactive GDP-bound Rac1 is removed during washing steps. The bound active Rac1 is detected with a Rac1 specific antibody. The degree of Rac1 activation is determined by comparing readings from activated lysates versus non-activated lysates. Inactivation of Rac1 is generally achieved in tissue culture by a serum starvation step (see Technical Guide). 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



Rac1 activation by EGF measured by G-LISA®. Swiss 3T3 (mouse) cells were serum starved for 48 hours and treated with EGF (Cat. # CN02; 10 ng/ml for 2 min). 25 µg of cell lysates were subjected to the G-LISA® assay. Absorbance was read at 490 nm. Data are background subtracted. Background values for this assay are in the range of 0.2--0.35.

II: Kit Contents

This kit contains enough reagents for 24 assays. When properly stored, kit components are guaranteed stable for a minimum of 6 months. You can assay anywhere from 2 to 24 samples at a time for your own convenience.

Table 1: Kit Contents

Reagents	Cat. # or Part # *	Quantity	Storage
Rac1-GTP strips	Part # GL551B	3 strips of 8 wells	Desiccated 4°C
Anti-Rac1 antibody (mouse monoclonal, Rac1 specific)	Part # GL07	1 tube, lyophilized	Desiccated 4°C
Secondary antibody - horseradish peroxidase conjugate (HRP)	Part # GL02	1 tube, lyophilized	Desiccated 4°C
Rac control protein (constitutively active Rac1)	Part # RCCA	3 tubes, lyophilized	Desiccated 4°C
Cell Lysis Buffer	Part # GL536	1 bottle, lyophilized	Desiccated 4°C
Wash Buffer	Part # PE38	1 tablet	Room temperature
Antigen Presenting Buffer	Part # GL545	1 bottle, 10 ml	Room temperature
Antibody Dilution Buffer	Part # GL540	1 bottle, lyophilized	Desiccated 4°C
HRP Detection Reagent A	Part # GL43	1 tablet, silver pack	Desiccated 4°C
HRP Detection Reagent B	Part # GL44	1 tablet, gold pack	Desiccated 4°C
HRP Stop Solution	Part # GL80	1 bottle, 8 ml	4°C
Precision Red™ Advanced Protein Assay Reagent	Part # GL550 (available as 500 ml size Cat. # ADV02)	1 bottle, 30 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)
- Concentrated sulfuric acid (need to add 1 ml to HRP Stop Buffer)
- Cell scrapers
- Liquid nitrogen for snap freezing cell lysates.
- Multi-channel or multi-dispensing pipette; 25-200 µl range.
- Multi-channel pipette solution basins (available from VWR Cat. # 21007-970). Used for liquid handling.
- Vortex for mixing cell lysate and other 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 spectrophotometer (see Technical Guide).

III: Reconstitution and Storage of Components

Many of the kit components are provided in lyophilized form. Prior to beginning the assay, you will need to reconstitute several components as shown in Table 2.

Table 2: Component Storage and Reconstitution

Kit Component	Reconstitution	Storage
Rac1-GTP strips	Keep the strips in the sealed bag with desiccant at all times. Reconstitution is not necessary prior to the start of the assay. If detached, pellets should be tapped to the bottom of the well prior to resuspension.	Store desiccated at 4°C
Anti-Rac1 antibody	Centrifuge briefly to collect the pellet to the bottom of the tube. Dissolve the powder in 40 µl of PBS.	Store at 4°C
Secondary antibody HRP	Centrifuge briefly to collect the pellet to 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
Rac control protein (3 tubes)	Each tube is good for one experiment. Reconstitution is not necessary until starting the assay (see Table 4).	Store desiccated at 4°C
Cell Lysis Buffer	Reconstitute in 30 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
Wash Buffer	Reconstitute tablet 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 5 ml of Milli-Q water.	Store at 4°C
HRP Detection Reagent A	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 12 x 0.8 ml volumes.	Store at -70°C NOTE: Do not store at –20°C
HRP Detection Reagent B	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 12 x 0.8 ml volumes.	Store at -70°C NOTE: Do not store at –20°C
HRP Stop Solution	Carefully add 1 ml of concentrated sulfuric acid (18 M) to HRP Stop Solution. Check the box on the top of the bottle to indicate acid has been added.	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

IV: Important Technical Notes

1. First time users should read the G-LISA Technical Guide, especially the description of lysate preparation which is critical for the success of the G-LISA assay (https://www.cytoskeleton.com/pdf-storage/info-res/glisa-technical-guide.pdf).

2. Changes made between manual Version 8.1 to manual v 10.0

    a. The production method for the G-LISA plate GL551 has been modified. This change has been denoted by a new Part # GL551B. Plate GL551B has a greater sensitivity to activated Rac1 in cell lysates.

    b. The primary antibody dilution has been changed from 1:50 to 1:300 to accommodate the greater sensitivity of the GL551B plate to activated Rac1.

    c. The amount of constitutively active Rac1-His protein has been increased from 3ng to 4 ng in the assay to enhance the robustness of the positive control signal. The positive control should give a raw reading of 0.9-1.3 at an OD of 490 nm.

First time users should read the G-LISA Technical Guide, especially the description of lysate preparation which is critical for the success of the G-LISA assay (https://www.cytoskeleton.com/pdf-storage/info-res/glisa-technical-guide.pdf). The G-LISA® kit uses 50 µl of lysate (0.25-1 mg/ml lysate protein concentration) per assay. To keep the assay in the linear range, we highly recommend using a lysate concentration of 0.25 - 1 mg/ml for the Rac1 G-LISA®.

A) Growth and Treatment of Cell Lines

Cells should be plated and grown to desired confluency in appropriate culture conditions (consult literature for particular cell line). Confluent cells can be used experimentally, including for transfection, RNA interference, or serum-starvation, if appropriate. Prior to Rac1 stimulation, cells should be kept in a "controlled state" via serum starvation so that basal Rac1 activity is low. Optimal confluency prior to serum starvation and GTPase activation varies by cell line and should be determined empirically. Upon stimulation, Rac proteins are generally activated very rapidly and transiently (30 s to 30 min).

B) Rapid Processing of Cells to Prepare Lysates

GTP bound (active) Rac1 is a labile entity and the bound GTP is susceptible to hydrolysis during and after cell lysis, resulting in Rac1 inactivation. Rapid processing (<10 min) on ice is essential for accurate and reproducible results. The following guidelines should be followed (See Table 3 for preparing reagents needed for cell lysate preparation).

Washing

1. Retrieve culture dish from incubator, immediately place on ice, aspirate off media, and wash cells with ice-cold PBS to remove serum proteins.

2. Aspirate off all PBS buffer. This is essential so that the lysis buffer is not diluted.

Cell Lysis

To make lysate at a concentration between 0.25 to 1.0 mg/ml, adjust the amount of lysis buffer depending on cell and plate type. Empirically determine the exact lysis volumes for any given cell line. The time period between cell lysis and snap-freezing of lysates is critically important (no more than 10 min on ice). Take the following precautions:

1. Keep solutions and lysates embedded in ice so that the temperature is below 4°C.

2. Lyse cells in an appropriate volume of ice-cold cell lysis buffer.

3. Immediately harvest cell lysates with a cell scraper.

4. Transfer lysates into pre-labeled and pre-chilled 1.5 ml microfuge tubes on ice.

5. Immediately clarify lysates by centrifugation at 10,000 x g , 4°C for 1 min.

6. Save at least 20 ul of lysate on ice for protein quantification and 50-200 ul for Rac1 quantification by western blotting.

7. Snap-freeze 120 µl aliquots of all cell lysates in liquid nitrogen immediately after harvest and clarification. Store at -70°C. Lysates can be stored at -70°C for no longer than 30 days.

C) Measure Lysate Protein Concentration

1. Add 20 µl of each lysate or lysis buffer into disposable 1 ml cuvettes.
2. Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Part # GL50) to each cuvette.
3. Incubate for 1 min at room temperature.
4. Blank spectrophotometer with the lysis buffer at 600 nm.
5. Read absorbance of lysates samples.
6. Multiply the absorbance by 5 to obtain the protein concentration in mg/ml (See Technical Guide).
7. Calculate how much ice-cold lysis buffer is needed to equalize the cell extracts to give identical protein concentrations in each sample between 0.25 - 1.0 mg/ml. It is not necessary to equalize protein concentration if the sample variation is less than 10%.

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

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

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

Technical Tip

Once an optimal protein concentration for the Rac1 assay has been determined, it is easier to equalize all cell lysates to the optimal concentration at the time of lysate preparation and before freezing lysate aliquots. This eliminates the need to equalize frozen lysate samples immediately prior to performing the assay and lysates can simply be thawed and used in the G-LISA.

Table 3: Reagents Needed for Lysate Preparation

✓	**Reagent**	**Preparation**
	Precision Red™ Advanced Protein Assay Reagent	Place on the bench and use at room temperature.
	1.5 ml microfuge tubes, labeled and chilled	Use for aliquoting lysates.
	Ice buckets	Use to pre-chill reagents and scrape cells.
	Protease Inhibitor Cocktail	Resuspend in 1 ml of dimethyl sulfoxide (DMSO) and keep at room temperature.
	Lysis buffer + protease inhibitors, ice-cold	a. Empirically determine volume of Lysis Buffer needed per culture vessel We recommend a final lysate concentration between 0.25-1.0 mg/ml for initial experiments. b. Determine **total volume** of Lysis Buffer needed by multiplying the lysis volume per culture vessel (µl) by number of vessels x 1.3. c. Aliquot this volume of Lysis Buffer into a clearly labeled tube and place in ice. d. Add 10 µl of protease inhibitor cocktail per ml of aliquoted Lysis Buffer. e. Mix well and leave on ice. f. Lysis Buffer needs to be **ice cold**.
	PBS pH 7.2, ice-cold	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.
	Cell scrapers	Use to harvest cells.
	Liquid nitrogen	Use to snap-freeze lysate aliquots.

IV: G-LISA Assay Protocol

First time users should read the G-LISA Technical Guide, especially the description of lysate preparation which is critical for the success of the G-LISA assay. The G-LISA® kit uses 50 µl of lysate (0.25-1 mg/ml protein concentration) per assay. To keep the assay in the linear range, we highly recommend using a lysate concentration of 0.25 - 1 mg/ml for the Rac1 G-LISA®. The reagents and equipment listed in Table 4 should be prepared prior to performing the assay.

Table 4: Assay Preparation for G-LISA®

✓	Reagent	Preparation
	Samples to be assayed	All experimental samples should be prepared prior to G-LISA assay preparation. The following instructions assume that frozen lysates are being used for the assay. Lysates should remain frozen until indicated in G-LISA assay protocol.
	Rac1-GTP binding strips	Remove strips from 4°C and keep in their protective bag. Place on your bench at room temperature for 30 min. Do not remove the strips from the bag until immediately prior to the experiment.
	Milli-Q water	30 ml placed on ice.
	Protease Inhibitor Cocktail	Resuspend in 1 ml of dimethyl sulfoxide (DMSO) and keep at room temperature.
	Lysis Buffer	Required for blank samples (60 µl per sample) and sample dilutions (if necessary). Add 10 µl of protease inhibitor cocktail per ml of Lysis Buffer. Mix well and leave on ice.
	Rac1 control protein	Dissolve one tube in 500 µl Lysis Buffer and leave on ice. Use within 15 minutes.
	Anti-Rac1 antibody	Have primary antibody stock ready on ice. For each 8-well strip, you will need to mix 1.7 µl antibody with 508.3 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 5 µl antibody with 500 µl Antibody Dilution Buffer. This dilution step should be performed just prior to use as detailed in assay protocol.
	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	The 0.8 ml aliquots of these reagents can remain at -70°C until secondary antibody addition as detailed in the assay protocol.
	HRP Stop Solution	Make sure that the box on top of the bottle is checked, indicating sulfuric acid has been added to the solution. Place the bottle on your bench and allow to warm to room temperature.
	Vortex	Used for mixing reagents, it is helpful to keep one close to the assay area.

1. Aliquot 120 µl of Lysis Buffer into a labeled microfuge tube. Place on ice. This is the buffer blank.

2. Mix 48 µl of RCCA (Rac1 Control Protein) with 72 µl of ice-cold Lysis Buffer in a labeled microfuge tube. Place on ice. This is the positive control sample. Do not re-use.

3. Remove the number of Rac1 strips required, place in strip holder, and place on ice. Return remaining strips to storage.

4. Keep the plate on ice and dissolve the powder in the wells with 100 µl ice-cold water. Detachment of the white powder pellet will not affect assay performance. Tap pellets to the bottom of the wells prior to resuspension.

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

6. If not already equalized, add required amount of ice-cold Lysis Buffer to equalize all lysate concentrations. Calculate dilution factors required BEFORE thawing lysates.

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

   Vigorously flick the plate to remove solution from each well, followed by a series of 5-7 vigorous pats onto paper towels. The complete removal of solution from wells between steps of the G-LISA® is very important to avoid high background readings. At an absorbance of 490 nm, buffer-only wells should read between 0.20 – 0.35 and positive control wells should read between 0.7-1.0 (after subtraction of blank).

8. Return plate back to ice. Immediately add 50 µl of equalized cell lysate to replicate wells.

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

10. Pipette 50 µl of Rac1 positive control into duplicate wells.

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

12. During the incubation, dilute the anti-Rac1 primary antibody to 1/300 in Antibody Dilution Buffer (add 1.7 µl of antibody to every 508.3 µl Antibody Dilution Buffer). Note: The final volume of 510 µl is adequate for one strip (8 wells).

13. After 30 min, remove the solution from the wells and wash twice with 200 µl Wash Buffer at room temperature using a multi-channel pipettor. Do not leave plate unattended at this time. Vigorously remove the Wash Buffer after each wash as described in step 7.

14. Place plate on the bench.

15. **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.**

16. Vigorously flick out the Antigen Presenting Buffer as described in step 7.

16. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer, removing Wash Buffer as described in step 7.

17. Add 50 µl of diluted anti-Rac1 primary antibody to each well and leave the plate on the orbital microplate shaker (200-400 rpm) at room temperature for 45 min.

18. During primary antibody incubation, dilute secondary HRP labeled antibody to 1/100 in Antibody Dilution Buffer (add 5 µl of antibody to every 500 µl Antibody Dilution Buffer). Note: The final volume of 500 µl is adequate for one strip (8 wells).

19. Vigorously flick out the anti-Rac1 primary antibody as described in step 7.

20. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer, removing Wash Buffer as described in step 7.

21. 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.

22. During secondary antibody incubation, thaw an aliquot of HRP detection reagents A and B in a room temperature water bath and remove as soon as they are thawed. Do not mix.

23. Immediately prior to the end of the secondary antibody incubation, mix HRP detection reagents A and B in equal volumes (50 µl of A/B mixture per well is needed). Protect from light. Discard unused solution.

24. Vigorously flick out the secondary antibody as described in step 7.

25. Wash the wells three times with 200 µl of room temperature Wash Buffer as described in step 7.

26. Pipette 50 µl of the mixed HRP detection reagent into each well and incubate at room temperature for 20 min.

27. Add 50 µl of HRP Stop Buffer to each well.

28. Check that the wells are free of bubbles; if not, remove before continuing.

29. Read the signal by measuring absorbance at 490 nm using a microplate spectrophotometer. Designate Lysis Buffer only wells as the assay Blank.

VI: Troubleshooting

Observation	Possible cause	Remedy
Weak or no signal in all wells.	1. Slow processing of samples or processing > 4°C. 2. Wells dried out during experiment. 3. Strips became damp during storage. Well contents will appear sticky and opaque. 4. A step or component of the assay was omitted. 5. Insufficient HRP reaction time.	1. Process samples quickly on ice. Snap freeze aliquots. 2. Do not remove the solution in the wells unless the next solution is ready. 3. Store the strips in the desiccant bag with the bag securely sealed. Keep the cover on the plate. If wells appear sticky and opaque, the plate can no longer be used. 4. Confirm with checklist that all reagents were added. 5. Develop for 20 min at room temperature. When Rac1 signal is very low, allow longer times (up to 30 min) for a stronger signal. HRP Stop Solution should be added prior to reading at 490 nm.
High signal in all wells.	1. Antibody concentration is too high. 2. Washes were insufficient.	1. Follow the recommended dilution of antibodies in the manual. If still too high, an antibody titration is necessary to optimize your results. 2. Follow the instructions for washing in the manual.
Background readings are high (>0.35).	1. Inefficient removal of solutions from G-LISA® wells.	1. Background should read between 0.20 – 0.35. Vigorous flicking and patting of the inverted plate is required to completely remove solutions from the wells after each step is complete. See G-LISA instructional video for details.
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-Rac1 level is too high. 5. Tissue culture cells not correctly serum starved. 6. Temperature of lysis and incubation is not 4°C. 7. The basal level of Rac1 is too high.	1. Purchase a fresh vial of inducer. 2. Confirm instructions were followed using the Experiment Record Sheet (see Technical Guide). 3. The linear range of the assay is 1 ng – 8 ng Rac1. 4. Titrate down the amount of extract to be added. 5. The Technical Guide and references therein give guidelines for serum starvation conditions. It is a good idea to stain cells with phalloidin to qualitatively determine success of serum starvation and induction. 6. Lyse cells on ice, keep cell lysis buffer and distilled water on ice. 7. Titrate controlled cell states (serum starved) as indicated in the protocol. If basal level reading is over 0.5 (after buffer blank subtraction), it is too high to detect correct activation ratio.
Significant variation between replicate samples.	1. Incorrect volume of solutions for each step added in the wells. 2. Inaccurate pipetting.	1. Follow the instruction for recommended volume in the manual. 2. A multi-channel pipettor is recommended.
Positive control not working.	1. Positive control protein was re-stored after reconstitution.	1. Use a fresh tube of Rac1 positive control protein each time. There are 3 per kit.

VI: Limited Use Statement

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.

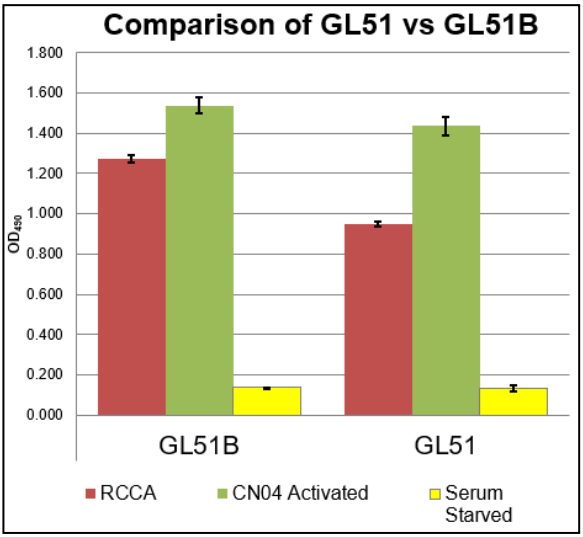
APPENDIX 1: GL551 & GL551B Comparison

Method

3T3 cells in DMEM media supplemented with 5% fetal bovine serum (FBS) were seeded onto tissue culture dishes (150 cm^2) and grown at 5% CO_2, 90% humidity, 37˚C to 50% confluency . Cells were subsequently serum starved for 24h. Half of the serum starved cells were treated for 2h with 1 µg/ml of a cell permeable catalytic domain of the bacterial cytotoxic necrotizing factor (Cat# CN04). This toxin has been shown to deamidate glutamine-61 of Rac1 and Cdc42 (and glutamine-63 of RhoA) which is in the Switch II region of the G-protein , thereby converting glutamine to glutamate, which blocks intrinsic and GAP-stimulated GTPase activity, resulting in constitutively active endogenous Rac1 (1). CN04 robustly increases the level of GTP-bound Rac1 within 2-4 h after addition to the culture medium. The other half of the cells were not treated with CN04 and were processed as shown below as serum starved lysates.

After CN04 treatment, both treated and untreated cells were harvested to give a final lysate concentration of 0.5 mg/ml. Lysates (50 ul/25 ug per assay) were assayed using either G-LISA plate GL51 or GL51B. All other reagents in the assay were identical except that the primary anti-Rac1 antibody (Part# GL07) was used at 1:50 dilution for GL51 plates and 1:300 dilution for GL51B plates. G-LISA assays were performed as described in this manual. Representative data for the comparative assays is given in Fig S1 below.

Figure S1: Comparison of G-LISA plates GL51 & GL51B for the detection of activated Rac1 protein in 3T3 cell lysates



Reference

1. Lerm M., et al. 1999. Deamidation of Cdc42 and Rac by Escherichia coli cytotoxic necrotizing factor 1: activation of c-Jun N-terminal kinase in HeLa cells. *Infection and immu-nity*. 67, 496-503.

NOTES

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V 12.0

G-LISA® Cdc42 Activation Assay Biochem Kit™: 24 Assays

(Absorbance Based)

Cat. # BK127-S

UPDATED FORMAT

Phone: (303) 322.2254 **Fax:** (303) 322.2257
Customer Service: cserve@cytoskeleton.com
Technical Support: tservice@cytoskeleton.com

Manual Contents

Assay Principle

The Cdc42 G-LISA® kit contains a Cdc42-GTP-binding protein linked to the wells of a 96 well plate. Active, GTP-bound Cdc42 in cell/tissue lysates will bind to the wells while inactive GDP-bound Cdc42 is removed during washing steps. The bound active Cdc42 is detected with a Cdc42 specific antibody. The degree of Cdc42 activation is determined by comparing readings from activated lysates versus non-activated lysates. Inactivation of Cdc42 is generally achieved in tissue culture by a serum starvation step (see G-LISA Technical Guide). 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

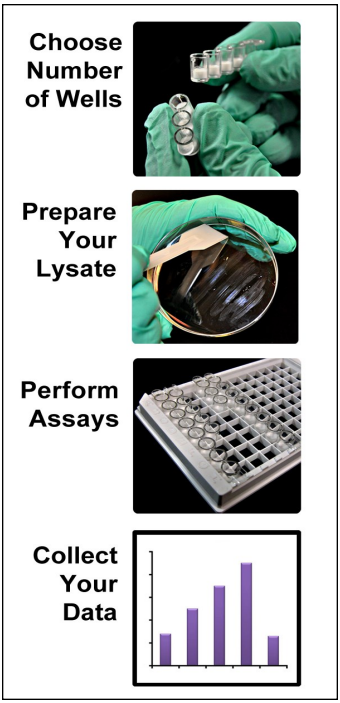
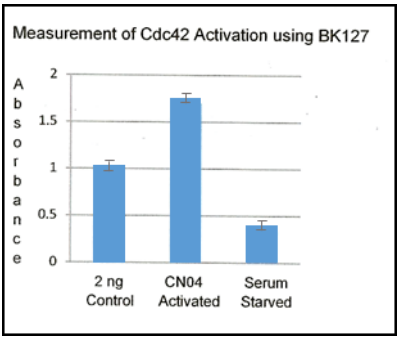


Figure 2: Typical G-LISA® Results



Cdc42 measurement using the BK127 (or BK127-S) G-LISA®.format. Control protein or cell lysates were incubated in separate wells of the G-LISA® plate. The wells were probed with anti-Cdc42 monoclonal antibody and a secondary antibody. Finally, the plate was developed with a colorimetric substrate and the absorbance was read at 490 nm. 2.5 ng control; constitutively active Cdc42 (Part # CDCA), CN04 activated; Swiss 3T3 lysates of cells activated with bacterial cytotoxic necrotizing factor toxin, serum starved; Swiss 3T3 lystaes of serum starved cells. Background values for this assay are 0.08-0.15. Data shown has been background subtracted.

II: Kit Contents

This kit contains enough reagents for 24 assays. When properly stored, kit components are guaranteed stable for a minimum of 6 months. You can assay anywhere from 2 to 24 samples at a time for your own convenience.

Table 1: Kit Contents

Reagents	Cat. # or Part # *	Quantity	Storage
Cdc42-GTP binding strips	Part # GL572B	3 strips of 8 wells	Desiccated 4°C
Anti-Cdc42 antibody (mouse monoclonal)	Part # GL514C	1 tube, lyophilized	Desiccated 4°C
Secondary antibody - horseradish peroxidase conjugate (HRP)	Part # GL02	1 tube, lyophilized	Desiccated 4°C
Cdc42 control protein (constitutively active Cdc42)	Part # CDCA	3 tubes, lyophilized	Desiccated 4°C
Cell Lysis Buffer	Part # GL535	1 bottle, lyophilized	Desiccated 4°C
Wash Buffer	Part # PE38	1 bottle, lyophilized	Desiccated 4°C
Antigen Presenting Buffer	Part # GL539	1 bottle, 10 ml	Room temperature
Antibody Dilution Buffer	Part # GL47	1 bottle, lyophilized	Desiccated 4°C
HRP Detection Reagent A	Part # GL43	1 tablet, silver pack	Desiccated 4°C
HRP Detection Reagent B	Part # GL44	1 tablet, gold pack	Desiccated 4°C
HRP Reagent Stop Solution	Part # GL80	1 bottle, 8 ml	4°C
Precision Red™ Advanced Protein Assay Reagent	Part # GL550 (available as 500 ml size Cat. # ADV02)	1 bottle, 30 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)
- Concentrated sulfuric acid (need to add 1 ml to HRP Stop Buffer)
- Cell scrapers
- Liquid nitrogen for snap freezing cell lysates.
- Multi-channel or multi-dispensing pipette; 25-200 µl range.
- Multi-channel pipette solution basins (available from VWR Cat. # 21007-970). Used for liquid handling.
- Vortex for mixing cell lysate and other 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 spectrophotometer (see Technical Guide)

III: Reconstitution and Storage of Components

Many of the kit components are provided in lyophilized form. Prior to beginning the assay, you will need to reconstitute several components as shown in Table 2.

Table 2: Component Storage and Reconstitution

Kit Component	Reconstitution	Storage
Cdc42-GTP binding strips	Keep the strips in the sealed bag with desiccant at all times. Reconstitution is not necessary prior to the start of the assay. If detached, pellets should be tapped to the bottom of the well prior to resuspension.	Store desiccated at 4°C
Anti-Cdc42 antibody	Centrifuge briefly to collect the pellet to the bottom of the tube. Dissolve the powder in 50 µl of PBS.	Store at 4°C
Secondary antibody HRP	Centrifuge briefly to collect the pellet to 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
Cdc42 control protein	This is resuspended in 500 ul of lysis buffer just prior to use. See Table 4. Each tube is good for one experiment.	Use within 15 min after reconstitution
Cell Lysis Buffer	Reconstitute in 30 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
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	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 12 x 0.8 ml volumes.	Store at -70°C NOTE: Do not store at –20°C
HRP Detection Reagent B	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 12 x 0.8 ml volumes.	Store at -70°C NOTE: Do not store at –20°C
HRP Stop Solution	Carefully add 1 ml of concentrated sulfuric acid (18 M) to HRP Stop Solution. Check the box on the top of the bottle to indicate acid has been added.	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

IV: Important Technical Notes and Manual Updates

1. First time users should read the G-LISA Technical Guide, especially the description of lysate preparation which is critical for the success of the G-LISA assay (https://www.cytoskeleton.com/pdf-storage/info-res/glisa-technical-guide.pdf).

2. Changes made between manual Version 11.0 to manual Version 12.0:

    a. The production method for the G-LISA plate GL572 has been modified. This change has been denoted by a new Part # GL572B. Plate GL572B has been extensively tested and developed to have a similar sensitivity to activated Cdc42 in cell lysates (see Appendix 1).

    b. A new Cdc42 primary antibody is used in this assay. This change has been denoted by a new Part # GL514C. Anti-Cdc42 antibody, GL514C, has been extensively tested and developed to have a similar sensitivity to activated Cdc42 in cell lysates (see Appendix 1).

    c. Primary antibody dilution has been adjusted from 1:20 to 1:80.

    d. Secondary antibody dilution has been adjusted from 1:62.5 to 1:100.

    e. HRP detection reagent volumes have been adjusted from 70 µl to 50 µl.

    f. STOP solution volumes have been adjusted from 140 µl to 50 µl.

V: How to Grow Cells and Prepare Lysates

First time users should read the G-LISA Technical Guide, especially the description of lysate preparation which is critical for the success of the G-LISA assay. The G-LISA® kit uses 50 µl of lysate (0.25-1 mg/ml lysate protein concentration) per assay. To keep the assay in the linear range, we highly recommend using a lysate concentration of 0.25 - 1 mg/ml for the Cdc42 G-LISA®.

A) Growth and Treatment of Cell Lines

Cells should be plated and grown to desired confluency in appropriate culture conditions (consult literature for particular cell line). Confluent cells can be used experimentally, including for transfection, RNA interference, or serum-starvation, if appropriate. Prior to Cdc42 stimulation, cells should be kept in a "controlled state" via serum starvation so that basal Cdc42 activity is low. Optimal confluency prior to serum starvation and GTPase activation varies by cell line and should be determined empirically. Upon stimulation, Cdc42 proteins are generally activated very rapidly and transiently (30 s to 30 min).

B) Rapid Processing of Cells to Prepare Lysates

GTP bound (active) Cdc42 is a labile entity and the bound GTP is susceptible to hydrolysis during and after cell lysis, resulting in Cdc42 inactivation. Rapid processing (<10 min) on ice is essential for accurate and reproducible results. The following guidelines should be followed (See Table 3 for preparing reagents needed for cell lysate preparation).

Washing

1. Retrieve culture dish from incubator, immediately place on ice, aspirate off media, and wash cells with ice-cold PBS to remove serum proteins.

2. Aspirate off all PBS buffer. This is essential so that the lysis buffer is not diluted.

Cell Lysis

To make lysate at a concentration between 0.25 to 1.0 mg/ml, adjust the amount of lysis buffer depending on cell and plate type. Empirically determine the exact lysis volumes for any given cell line. The time period between cell lysis and snap-freezing of lysates is critically important (no more than 10 min on ice). Take the following precautions:

1. Keep solutions and lysates embedded in ice so that the temperature is below 4°C.

2. Lyse cells in an appropriate volume of ice-cold cell lysis buffer.

3. Immediately harvest cell lysates with a cell scraper.

4. Transfer lysates into pre-labeled and pre-chilled 1.5 ml microfuge tubes on ice.

5. Immediately clarify lysates by centrifugation at 10,000 x g , 4°C for 1 min.

6. Save at least 20 ul of lysate on ice for protein quantification and 50-200 µl for Cdc42 quantification by western blotting.

6. Snap-freeze 120 µl aliquots of all cell lysates in liquid nitrogen immediately after harvest and clarification. Store at -70°C. Lysates can be stored at -70°C for no longer than 30 days.

C) Measure Lysate Protein Concentration

1. Add 20 µl of each lysate or lysis buffer into disposable 1 ml cuvettes.
2. Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Part # GL550) to each cuvette.
3. Incubate for 1 min at room temperature.
4. Blank spectrophotometer with the lysis buffer at 600 nm.
5. Read absorbance of lysates samples.
6. Multiply the absorbance by 5 to obtain the protein concentration in mg/ml (See Technical Guide).
7. Calculate how much ice-cold lysis buffer is needed to equalize the cell extracts to give identical protein concentrations in each sample between 0.25 - 1.0 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:

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

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

Technical Tip

Once an optimal protein concentration for the Cdc42 assay has been determined, it is easier to equalize all cell lysates to the optimal concentration at the time of lysate preparation and before freezing lysate aliquots. This eliminates the need to equalize frozen lysate samples immediately prior to performing the assay and lysates can simply be thawed and used in the G-LISA.

Table 3: Reagents Needed for Lysate Preparation

✓	**Reagent**	**Preparation**
	Precision Red™ Advanced Protein Assay Reagent	Place on the bench and use at room temperature.
	1.5 ml microfuge tubes, labeled and chilled	Use for aliquoting lysates.
	Ice buckets	Use to pre-chill reagents and scrape cells.
	Protease Inhibitor Cocktail	Resuspend in 1 ml of dimethyl sulfoxide (DMSO) and keep at room temperature.
	Lysis buffer + protease inhibitors, ice-cold	a. Empirically determine volume of Lysis Buffer needed per culture vessel (see Technical Guide**)**. We recommend a final lysate concentration between 0.25-1.0 mg/ml for initial experiments. b. Determine **total volume** of Lysis Buffer needed by multiplying the lysis volume per culture vessel (µl) by number of vessels x 1.3. c. Aliquot this volume of Lysis Buffer into a clearly labeled tube and place in ice. d. Add 10 µl of protease inhibitor cocktail per ml of aliquoted Lysis Buffer. e. Mix well and leave on ice. f. Lysis Buffer needs to be **ice cold**.
	PBS pH 7.2, ice-cold	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.
	Cell scrapers	Use to harvest cells.
	Liquid nitrogen	Use to snap-freeze lysate aliquots.

V: G-LISA Assay Preparation

First time users should read the G-LISA Technical Guide, especially the description of lysate preparation which is critical for the success of the G-LISA assay. The G-LISA® kit uses 50 µl of lysate (0.25-1 mg/ml protein concentration) per assay. To keep the assay in the linear range, we highly recommend using a lysate concentration of 0.25 - 1 mg/ml for the Cdc42 G-LISA®. The reagents and equipment listed in Table 4 should be prepared prior to performing the assay.

Table 4: Assay Preparation for G-LISA®

✓	Reagent	Preparation
	Samples to be assayed	All experimental samples should be prepared prior to G-LISA assay preparation. The following instructions assume that frozen lysates are being used for the assay. Lysates should remain frozen until indicated in G-LISA assay protocol.
	Cdc42-GTP binding strips	Remove strips from 4°C and keep in its protective bag. Place on your bench at room temperature for 30 min. Do not remove the strips from the bag until immediately prior to the experiment.
	Milli-Q water	30 ml placed on ice.
	Protease Inhibitor Cocktail	Resuspend in 1 ml of dimethyl sulfoxide (DMSO) and keep at room temperature.
	Lysis Buffer	Required for blank samples (60 µl per sample) and sample dilutions (if necessary). Add 10 µl of protease inhibitor cocktail per ml of Lysis Buffer. Mix well and leave on ice.
	Cdc42 control protein	Dissolve one tube in 500 µl Lysis Buffer and leave on ice. Use within 15 minutes. Discard any unused stock.
	Anti-Cdc42 antibody	Have primary antibody stock ready on ice. For each 8-well strip, you will need to mix 6.3 µl antibody with 500 µl Antibody Dilution Buffer (1:80 dilution). 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 5 µl antibody with 500 µl Antibody Dilution Buffer (1:100 dilution). This dilution step should be performed just prior to use as detailed in assay protocol.
	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	The 0.8 ml aliquots of these reagents can remain at -70°C until secondary antibody addition as detailed in the assay protocol.
	HRP Stop Solution	Make sure that the box on top of the bottle is checked, indicating sulfuric acid has been added to the solution. Place the bottle on your bench and allow to warm to room temperature.
	Vortex	Used for mixing reagents, it is helpful to keep one close to the assay area.

V: G-LISA Assay Protocol

1. Aliquot 120 µl of Lysis Buffer into a labeled microfuge tube. Place on ice. This is the buffer blank.

2. Mix 30 µl of CDCA (Cdc42 Control Protein) with 90 µl of ice-cold Lysis Buffer in a labeled microfuge tube. Place on ice. This is the positive control sample. The positive control is at 2.5ng per well and should be 1-1.2 after background subtraction. Do not re-use.

3. Remove the number of Cdc42-GTP strips required, place in strip holder, and place on ice. Return remaining strips to storage.

4. Keep the plate on ice and dissolve the powder in the wells with 100 µl ice-cold water. Detachment of the whilte powder pellet will not affect assay performance. Tap pellets to the bottom of the wells prior to resuspension.

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

6. If not already equalized, add required amount of ice-cold Lysis Buffer to equalize all lysate concentrations. Calculate dilution factors required BEFORE thawing lysates.

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

   Vigorously flick the plate to remove solution from each well, followed by a series of 5-7 vigorous pats onto paper towels. The complete removal of solution from wells between steps of the G-LISA® is very important to avoid high background readings. At an absorbance of 490 nm, buffer-only wells should read between 0.08 – 0.15.

8. Return plate back to ice. Immediately add 50 µl of equalized cell lysate to replicate wells.

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

10. Pipette 50 µl of Cdc42 positive control into duplicate wells.

11. Immediately place the plate on a cold orbital microplate shaker (400 rpm recommended, 200 rpm minimum) at 4°C for exactly 15 min.

12. During the incubation, dilute the anti-Cdc42 primary antibody to 1/80 in Antibody Dilution Buffer (add 6.3 µl of antibody to every 500 µl Antibody Dilution Buffer). Note: The final volume of 506.3 µl is adequate for one strip (8 wells).

13. After 15 min, remove the solution from the wells and wash twice with 200 µl Wash Buffer at room temperature using a multi-channel pipettor. Do not leave plate unattended at this time. Vigorously remove the Wash Buffer after each wash as described in step 7.

14. Place plate on the bench.

15. **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.**

16. Vigorously flick out the Antigen Presenting Buffer as described in step 7.

17. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer, removing Wash Buffer as described in step 7.

18. Add 50 µl of diluted anti-Cdc42 primary antibody to each well and leave the plate on the orbital microplate shaker (200-400 rpm) at room temperature for 30 min.

19. During primary antibody incubation, dilute secondary HRP labeled antibody to 1/100 in Antibody Dilution Buffer (add 5 µl of antibody to every 500 µl Antibody Dilution Buffer). Note: The final volume of 505 µl is adequate for one strip (8 wells).

20. Vigorously flick out the anti-Cdc42 primary antibody as described in step 7.

21. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer, removing Wash Buffer as described in step 7.

22. 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 30 min.

23. During secondary antibody incubation, thaw an aliquot of HRP detection reagents A and B in a room temperature water bath and remove as soon as they are thawed. Do not mix.

24. Immediately prior to the end of the secondary antibody incubation, mix HRP detection reagents A and B in equal volumes (50 µl of A/B mixture per well is needed). Protect mixture from light. Discard unused solution.

25. Vigorously flick out the secondary antibody as described in step 7.

26. Wash the wells three times with 200 µl of room temperature Wash Buffer as described in step 7.

27. Pipette 50 µl of the mixed HRP detection reagent into each well and incubate at room temperature for 15 min.

28. Add 50 µl of HRP Stop Buffer to each well.

29. Check that the wells are free of bubbles; if not, remove before continuing.

30. Immediately read the signal by measuring absorbance at 490 nm using a microplate spectrophotometer. Designate Lysis Buffer only wells as the assay Blank.

VI: Troubleshooting

Observation	Possible cause	Remedy
Weak or no signal in all wells.	1. Slow processing of samples or processing > 4°C.	1. Process samples quickly on ice. Snap freeze aliquots.
	2. Wells dried out during experiment.	2. Do not remove the solution in the wells unless the next solution is ready.
	3. Strips became damp during storage. Well contents will appear sticky and opaque.	3. Store the strips in the desiccant bag with the bag securely sealed. If wells appear sticky and opaque, the strips can no longer be used.
	4. A step or component of the assay was omitted.	4. Confirm with checklist that all reagents were added.
	5. Insufficient HRP reaction time.	5. When Cdc42 signal is very low, allow longer HRP development times for a stronger signal. HRP Stop Solution should be added prior to reading at 490 nm.
High signal in all wells.	1. Antibody concentration is too high.	1. Follow the recommended dilution of antibodies in the manual. If still too high, an antibody titration is necessary to optimize your results.
	2. Washes were insufficient.	2. Follow the instructions for washing in the manual.
	3. HRP reagents stored incorrectly	3. Aliquot HRP solutions and store at –70° C. Do not reuse aliquots once thawed.
Background readings are high (>0.40).	1. Inefficient removal of solutions from G-LISA® wells.	1. Background should read between 0.08 – 0.15. Vigorous flicking and patting of the inverted plate is required to completely remove solutions from the wells after each step is complete. See G-LISA instructional video for details.
	2. HRP reagents stored incorrectly	2. Aliquot HRP solutions and store at –70° C. Do not reuse aliquots once thawed
Induced sample does not give significant signal increase.	1. Poor inducer activity.	1. Purchase a fresh vial of inducer.
	2. Technique not rapid or cold enough.	2. Confirm instructions were followed using the Experiment Record Sheet (see Technical Guide).
	3. The endogenous GTP-Cdc42 level is too high.	3. Titrate down the amount of extract to be added.
	4. Tissue culture cells not correctly serum starved.	4. See Technical Guide and references therein for guidance on serum starvation. It is a good idea to stain cells with phalloidin to qualitatively determine success of serum starvation and induction.
	5. Temperature of lysis and incubation is not 4°C.	5. Lyse cells on ice, keep cell lysis buffer and distilled water on ice.
Significant variation between replicate samples.	1. Incorrect volume of solutions for each step added in the wells.	1. Follow the instruction for recommended volume in the manual.
	2. Inaccurate pipetting.	2. A multi-channel pipettor is recommended.
Positive control not working.	1. Positive control protein was stored incorrectly after reconstitution.	1. Store at 4°C and use within 15 minutes. Discard unused control as it is not stable.
	2. Positive control resuspended incorrectly	

VI: Limited Use Statement

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.

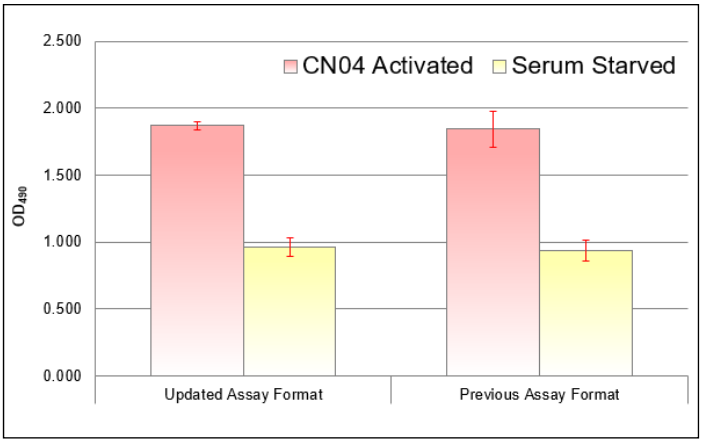
VII: Appendix 1: Updated Assay Format Compared to Previous Assay Format

Method

3T3 cells in DMEM media supplemented with 5% fetal bovine serum (FBS) were seeded onto tissue culture dishes (150 cm^2) and grown at 5% CO_2, 90% humidity, 37˚C to 50% confluency . Cells were subsequently serum starved for 24h. Half of the serum starved cells were treated for 2h with 1 µg/ml of a cell permeable catalytic domain of the bacterial cytotoxic necrotizing factor (Cat# CN04). This toxin has been shown to deamidate glutamine-61 of Cdc42 and Rac1 (and glutamine-63 of RhoA) which is in the Switch II region of the G-protein , thereby converting glutamine to glutamate, which blocks intrinsic and GAP-stimulated GTPase activity, resulting in constitutively active endogenous Cdc42 (1). CN04 robustly increases the level of GTP-bound Cdc42 within 2-4 h after addition to the culture medium. The other half of the cells were not treated with CN04 and were processed as shown below as serum starved lysates.

After CN04 treatment, both treated and untreated cells were harvested to give a final lysate concentration of 0.25 mg/ml. Lysates (50 ul/12.5 ug per assay) were assayed using either the updated assay format (as described in this manual and see Section IV) or the previous assay format (as detailed in manuals prior to version 12.0 and see Section IV). Representative data for the comparative assays is given in Fig S1 below.

Figure S1: Comparison of G-LISA assay formats (updated vs previous) for the detection of activated Cdc42 protein in 3T3 cell lysates



Reference

1. Lerm M., et al. 1999. Deamidation of Cdc42 and Rac by Escherichia coli cytotoxic necrotizing factor 1: activation of c-Jun N-terminal kinase in HeLa cells. *Infection and immu-nity*. 67, 496-503.

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V. 17.0

G-LISA® RhoA Activation Assay

Biochem Kit™: 24 Assays

(Absorbance Based)

Cat. # BK124-S

UPDATED FORMAT

Phone: (303) 322.2254 **Fax:** (303) 322.2257
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Manual Contents

Assay Principle

The RhoA G-LISA® kit contains a Rho GTP-binding protein linked to the wells of strips from 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. The degree of RhoA activation is determined by comparing readings from activated versus non-activated lysates. Inactivation of RhoA is generally achieved in tissue culture by a serum starvation step (see Technical Guide). 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

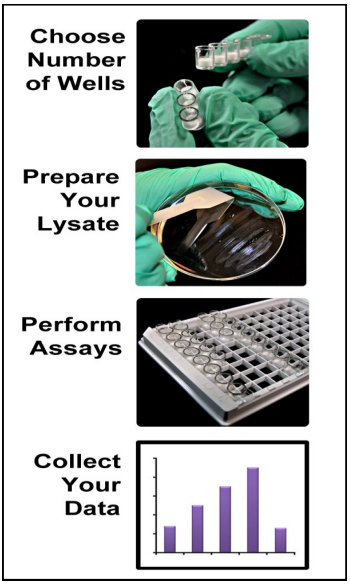
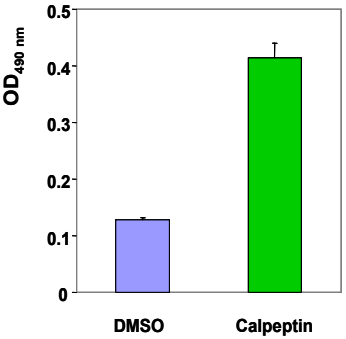


Figure 2: Typical G-LISA® Results



RhoA activation by calpeptin measured by G-LISA®. Swiss 3T3 (mouse) cells were serum starved for 48 hours and treated with calpeptin (Cat. # CN01; 0.1 mg/ml for 30 min) or carrier only (DMSO). 25 µg of cell lysates were subjected to the G-LISA® assay. Absorbance was read at 490 nm. Data are background subtracted. Background values for this assay are 0.1-0.25 OD.

II: Kit Contents

This kit contains enough reagents for 24 assays. When properly stored, kit components are guaranteed stable for a minimum of 6 months.

Table 1: Kit Contents

Reagents	Cat. # or Part # *	Quantity	Storage
Rho-GTP binding strips	Part # GL525B	3 strips of 8 wells	Desiccated 4°C
Anti-RhoA antibody	Part # GL01C	1 tube, 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	3 tubes, lyophilized	Desiccated 4°C
Cell Lysis Buffer	Part # GL536	1 bottle, lyophilized	Desiccated 4°C
Binding Buffer	Part # GL37	1 bottle, lyophilized	Desiccated 4°C
Wash Buffer	Part # PE38	1 tablet	4°C
Antigen Presenting Buffer	Part # GL545	1 bottle, 10 ml	Room temperature
Antibody Dilution Buffer	Part # GL540	1 bottle, lyophilized	Desiccated 4°C
HRP Detection Reagent A	Part # GL43	1 tablet, silver pack	Desiccated 4°C
HRP Detection Reagent B	Part # GL44	1 tablet, gold pack	Desiccated 4°C
HRP Stop Solution	Part # GL80	1 bottle, 8 ml	4°C
Precision Red™ Advanced Protein Assay Reagent	Part # GL550 (available as 500 ml size Cat. # ADV02)	1 bottle, 30 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 pH 7.2 buffer (10 mM phosphate buffer pH 7.4, 140 mM NaCl, 3 mM KCl)
- Concentrated sulfuric acid (need to add 1 ml to HRP Stop Buffer)
- 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 spectrophotometer (see Technical Guide: www.cytoskeleton.com/pdf-storage/info-res/glisa-technical-guide.pdf).

III: Reconstitution and Storage of Components

Many of the kit components are provided in lyophilized form. Prior to beginning the assay, you will need to reconstitute several components as shown in Table 2:

Table 2: Component Storage and Reconstitution

Kit Component	Reconstitution	Storage Conditions
Rho-GTP binding strips	Keep the strips in the sealed bag with desiccant at all times. Reconstitution is not necessary prior to the start of the assay. If detached, 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. For each tube, dissolve the powder in 20 µl of PBS.	Store at 4°C
Secondary antibody HRP	Centrifuge briefly to collect the pellet in the bottom of the tube. For each 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 (3 tubes)	Each tube is good for one experiment. Reconstitution is not necessary until starting the assay (see Table 4).	Store desiccated at 4°C
Cell Lysis Buffer	Reconstitute in 30 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 tablet 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 5 ml of Milli-Q water.	Store at 4°C
HRP Detection Reagent A	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 10 x 1 ml volumes.	Store at -70°C NOTE: Do not store at -20°C
HRP Detection Reagent B	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 10 x 1 ml volumes.	Store at -70°C NOTE: Do not store at -20°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
HRP Stop Solution	Carefully add 1 ml of concentrated sulfuric acid (18M) to HRP Stop Solution. Check the box on the top of the bottle to indicate acid has been added.	Store at 4°C

IV: Important Technical Notes & Manual Updates

1. First time users should read the G-LISA Technical Guide, especially the description of lysate preparation which is critical for the success of the G-LISA assay (https://www.cytoskeleton.com/pdf-storage/info-res/glisa-technical-guide.pdf).

2. Changes made to manual Version 16.0:17.0:

    a. The production method for the G-LISA plate GL25 has been modified. This change has been denoted by a new Part # GL25B. Plate GL25B has been extensively tested and developed to have a similar sensitivity to activated Rho in cell lysates (see Appendix 1).

    b. GL01B has been updated to double the amount of antibody per tube and has been given an updated part # GL01C. The increased amount of antibody gives a more robust signal.

V: How to Grow Cells and Prepare Lysates

First time users should read the G-LISA Technical Guide (www.cytoskeleton.com/pdf-storage/info-res/glisa-technical-guide.pdf) for a description of lysate processing from tissue and 3D cell culture samples. The description below is for 2D tissue culture samples. The G-LISA® kit uses 25 µl of lysate (0.4-2 mg/ml lysate protein concentration) per assay. To keep the assay in the linear range, we highly recommend using a lysate concentration of 0.5 mg/ml for the RhoA G-LISA®.

A) Growth and Treatment of Cell Lines

Cells should be plated and grown to desired confluency in appropriate culture conditions (consult literature for particular cell line). Confluent cells can be used experimentally, including for transfection, RNA interference, or serum-starvation, if appropriate. Prior to Rho stimulation, cells should be kept in a "controlled state" via serum starvation so that basal Rho activity is low. Optimal confluency prior to serum starvation and GTPase activation varies by cell line and should be determined empirically. Upon stimulation, Rho proteins are generally activated very rapidly and transiently (30 s to 30 min).

B) Rapid Processing of Cells to Prepare Lysates

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 (<10 min) on ice is essential for accurate and reproducible results. The following guidelines should be followed (See Table 3 for preparing reagents needed for cell lysate preparation).

Washing

1. Retrieve culture dish from incubator, immediately place on ice, aspirate off media, and wash cells with ice-cold PBS to remove serum proteins.

2. Aspirate off all PBS buffer. This is essential so that the lysis buffer is not diluted.

Cell Lysis

To make lysate at a concentration between 0.4 to 2.0 mg/ml, adjust the amount of lysis buffer depending on cell and plate type. Empirically determine the exact lysis volumes for any given cell line. The time period between cell lysis and snap-freezing of lysates is critically important (no more than 10 min on ice). Take the following precautions:

1. Keep solutions and lysates embedded in ice so that the temperature is below 4°C.

2. Lyse cells in an appropriate volume of ice-cold cell lysis buffer.

3. Immediately harvest cell lysates with a cell scraper.

4. Transfer lysates into pre-labeled and pre-chilled 1.5 ml microfuge tubes on ice.

5. Immediately clarify lysates by centrifugation at 10,000 x g , 4°C for 1 min.

6. Save at least 20 µl of lysate on ice for protein quantification and 50-200 µl for RhoA quantification by western blotting or the Total RhoA ELISA (Cat. # BK150).

6. Snap-freeze 100 µl aliquots of all cell lysates in liquid nitrogen immediately after harvest and clarification. Store at -70°C. Lysates can be stored at -70°C for several months.

C) Measure Lysate Protein Concentration

1. Add 20 µl of each lysate or lysis buffer into disposable 1 ml cuvettes.
2. Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Part # GL50) to each cuvette.
3. Incubate for 1 min at room temperature.
4. Blank spectrophotometer with the lysis buffer at 600 nm.
5. Read absorbance of lysates samples.
6. Multiply the absorbance by 5 to obtain the protein concentration in mg/ml (see Technical Guide).
7. Calculate how much ice-cold lysis buffer is needed to equalize the cell extracts to give identical protein concentrations in each sample between 0.4-2.0 mg/ml. It is not necessary to equalize protein concentration if the sample variation is less than 10%.

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

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

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

Technical Tip

Once an optimal protein concentration for the RhoA assay has been determined, it is easier to equalize all cell lysates to the optimal concentration at the time of lysate preparation and before freezing lysate aliquots. This eliminates the need to equalize frozen lysate samples immediately prior to performing the assay and lysates can simply be thawed and used in the G-LISA.

Table 3: Reagents Needed for Lysate Preparation

✓	Reagent	Preparation
	Precision Red™ Advanced Protein Assay Reagent	Place on the bench and use at room temperature.
	1.5 ml microfuge tubes, labeled and chilled	Use for aliquoting lysates.
	Ice buckets	Use to pre-chill reagents and scrape cells.
	Protease Inhibitor Cocktail	Resuspend in 1 ml of dimethyl sulfoxide (DMSO) and keep at room temperature.
	Lysis buffer + protease inhibitors, ice-cold	a. Empirically determine volume of Lysis Buffer needed per culture vessel (see Technical Guide). We recommend a final lysate concentration between 0.4-2.0 mg/ml for initial experiments. b. Determine **total volume** of Lysis Buffer needed by multiplying the lysis volume per culture vessel (µl) by number of vessels x 1.3. c. Aliquot this volume of Lysis Buffer into a clearly labeled tube and place in ice. d. Add 10 µl of protease inhibitor cocktail per ml of aliquoted Lysis Buffer. e. Mix well and leave on ice. f. Lysis Buffer needs to be **ice cold**.
	PBS pH 7.2, ice-cold	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.
	Cell scrapers	Use to harvest cells.
	Liquid nitrogen	Use to snap-freeze lysate aliquots.

V: G-LISA Assay Preparation

First time users should read the G-LISA Technical Guide (https://www.cytoskeleton.com/pdf-storage/info-res/glisa-technical-guide.pdf), especially the description of lysate preparation which is critical for the success of the G-LISA assay. The G-LISA® kit uses 25 µl of lysate (0.4-2 mg/ml lysate protein concentration) per assay. To keep the assay in the linear range, we highly recommend using a lysate concentration of 0.5 mg/ml for the RhoA G-LISA®. The reagents and equipment listed in **Table 4** should be prepared prior to performing the assay.

Table 4: Assay Preparation for G-LISA®

✓	**Reagent**	**Preparation**
	Samples to be assayed	All experimental samples should be prepared prior to G-LISA assay preparation. The following instructions assume that frozen lysates are being used for the assay. Lysates should remain frozen until indicated in G-LISA
	Rho-GTP binding Strips	Remove strips from 4°C. Keep in their protective bag. Place on bench at room temperature for 30 min. Do not remove the 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	Required for blank samples (60 µl per sample) and sample dilutions (if necessary) Add 10 µl of protease inhibitor cocktail per ml of Lysis Buffer. Mix well and leave on ice.
	Rho control protein	Dissolve one tube in 500 µl Lysis Buffer and leave on ice. Use within 15 minutes.
	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 8 µl antibody with 500 µl Antibody Dilution Buffer. This dilution step should be performed just prior to use as detailed in assay protocol.
	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	The 1 ml aliquots of these reagents can remain at -70°C until secondary antibody addition as detailed in the assay protocol.
	HRP Stop Solution	Make sure that the box on top of the bottle is checked, indicating sulfuric acid has been added to the solution. Place the bottle on your bench and allow to warm to room temperature.
	Vortex	Used for mixing reagents, it is helpful to keep one close to the assay area.

V: G-LISA Assay Protocol

1. Mix 60 µl Lysis Buffer with 60 µl ice-cold Binding Buffer. Place on ice. This is the buffer blank.

2. Mix 12 µl Rho Control Protein with 48 µl Cell Lysis Buffer and 60 µl Binding Buffer. Place on ice. This is the positive control sample. Do not re-use.

3. Remove the number of Rho strips required, place in strip holder, and place on ice. Return remaining strips to storage.

4. Keep the plate on ice and dissolve the powder in the wells with 100 µl ice-cold water. Detachment of the white powder pellet will not affect assay performance. Tap pellets to the bottom of the wells prior to resuspension.

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

6. If not already equalized, add required amount of ice-cold lysis buffer to equalize all lysate concentrations. Calculate dilution factors required BEFORE thawing lysates.

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 4°C Binding Buffer to each tube. Vortex each tube for 3-5 s on a high setting and return tubes to ice.

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

   Vigorously flick the plate to remove solution from each well, followed by a series of 5-7 vigorous pats onto paper towels. The complete removal of solution from wells between steps of the G-LISA is very important to avoid high background readings. At an absorbance of 490 nm, buffer-only wells should read between 0.10 – 0.25 and positive control wells should read between 0.7 – 1.3 (after subtraction of blank).

10. Return plate to ice. Immediately add 50 µl of equalized cell lysate to wells.

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

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

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

14. During the incubation, dilute anti-RhoA primary antibody to 1/250 in Antibody Dilution Buffer (add 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).

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

16. Place plate on the bench.

17. **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.**

18. Vigorously flick out the Antigen Presenting Buffer as described in step 9.

19. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer as described in step 9.

20. 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.

21. During primary antibody incubation, dilute the secondary HRP labeled antibody to 1/62.5 in Antibody Dilution Buffer (add 8 µl of antibody to every 500 µl Antibody Dilution Buffer). Note: The final volume of 500 µl is adequate for one strip (8 wells).

22. Vigorously flick out the anti-RhoA primary antibody as described in step 9.

23. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer as described in step 9.

24. 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.

25. During secondary antibody incubation, thaw an aliquot of HRP detection reagents A and B in a room temperature water bath and remove as soon as they are thawed. Do not mix.

26. Immediately prior to the end of the secondary antibody incubation, mix HRP detection reagents A and B in equal volumes (50 µl of A/B mixture per well is needed). Protect from light. Discard unused solution.

27. Vigorously flick out the secondary antibody as described in step 9.

28. Wash the wells three times with 200 µl of room temperature Wash Buffer as described in step 9.

29. Pipette 50 µl of the mixed HRP detection reagent into each well and incubate at 37°C for 10-15 min.

30. Add 50 µl of HRP Stop Buffer.

31. Check that the wells are free of bubbles; if not, remove before continuing.

32. Read the signal by measuring absorbance at 490 nm using a microplate spectrophotometer. Designate Lysis Buffer only wells as the assay Blank.

VI: Troubleshooting

Observation	Possible cause	Remedy
Weak or no signal in all wells.	1. Slow processing of samples or processing > 4°C. 2. Wells dried out during experiment. 3. Strips became damp during storage. Well contents will appear sticky and opaque. 4. A step or component of the assay was omitted. 5. Insufficient HRP reaction time.	1. Process samples quickly on ice. Snap freeze aliquots. 2. Do not remove the solution in the wells unless the next solution is ready. 3. Store the strips in the desiccant bag with the bag securely sealed. Keep the cover on the plate. If wells appear sticky and opaque, the plate can no longer be used. 4. Confirm with checklist that all regents were added. 5. Develop for 10-15 min at 37°C. HRP Stop Solution should be added prior to reading at 490 nm.
High signal in all wells.	1. Antibody concentration is too high. 2. Washes were insufficient.	1. Follow the recommended dilution in protocol. If still too high, an antibody titration is necessary to optimize your results. 2. Follow washing protocol.
High background readings (>0.25).	1. Inefficient removal of solutions from G-LISA wells.	1. Background should read between 0.10 – 0.25. Vigorous flicking and patting of the inverted plate is required to completely remove solutions from the wells after each step is complete. See G-LISA instructional video for details.
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. Confirm instructions were followed using the Experiment Record Sheet (see Technical Guied for record sheet). 3. The linear range of the assay is 0.05 – 2 ng RhoA. 4. Titrate down the amount of extract to be added. 5. See Technical Guide and references therein for guidance on serum starvation. It is a good idea to stain cells with phalloidin to qualitatively determine success of serum starvation and induction. 6. Lyse cells on ice and keep all cell lysis reagents on ice. 7. Make sure the buffer was stored in the fridge and kept on ice before use.
Significant variation between replicate samples.	1. Incorrect volume of solutions for each step added in the wells. 2. Inaccurate pipetting. 3. Did not vortex lysates after Binding Buffer addition.	1. Follow the instruction for recommended volume in the manual. 2. Use a multi-channel pipettor. 3. Binding Buffer is viscous and requires a vortex step to mix efficiently with lysate.
Positive control not working.	1. Positive control protein was re-stored after reconstitution.	1. Use a fresh tube of RhoA positive control protein each time. There are 3 per kit.

VI: Limited Use Statement

Limited Use Statement

The G-LISA® kits are based on technology developed at Cytoskeleton Inc. and are the subject of patent applications assigned to 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.

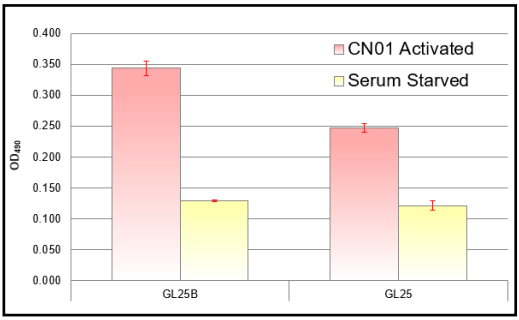
The terms of this Limited Use Statement apply to all buyers including academic and for-profit entities. If the purchaser is not willing to accept the conditions of this Limited Use Statement, Cytoskeleton Inc. is willing to accept return of the unused product with a full refund.

Method

3T3 cells in DMEM media supplemented with 5% fetal bovine serum (FBS) were seeded onto tissue culture dishes (150 cm^2) and grown at 5% CO_2, 90% humidity, 37˚C to 30-40% confluency . Cells were subsequently serum starved for 24-48h. Half of the serum starved cells were treated for 20 minutes with 0.1 mg/ml of calpeptin (Cat# CN01). The remaining half of the cells remained untreated and were processed as Serum Starved lysates. Calpeptin has been shown to activate Rho A, B and C indirectly via a mechanism resulting in constitutive activation of Rho GEFs through inhibition of the tyrosine phosphatase Shp-2 (1).

After CN01 treatment, both treated and untreated cells were harvested to give a final lysate concentration of 0.5 mg/ml. Lysates (50 ul/25 ug per assay) were assayed using either G-LISA plate GL25 or GL25B (Note: GL525/B are identical to GL25/B plates, only the quantity of wells per kit differs). All other reagents in the assay were identical. G-LISA assays were performed as described in this manual. Representative data for the comparative assays is given in Fig S1 below.

Figure S1: Comparison of G-LISA plates GL25 & GL25B for the detection of activated RhoA protein in 3T3 cell lysates



Reference

1. Schoenwaelder S.M. et al. 2000. The protein tyrosine phosphatase Shp-2 regulates RhoA activity. *Current Biol.* **10**, 1523-1526.

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