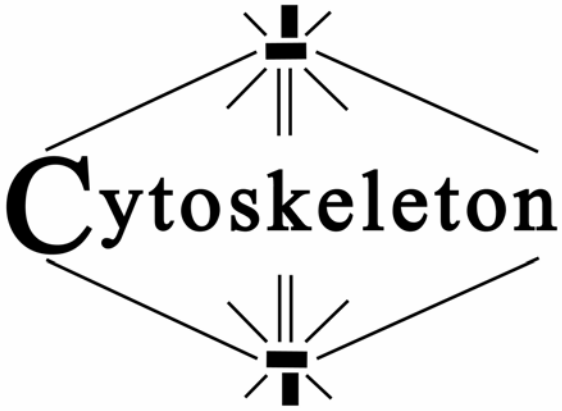

G-LISA™ RhoA Activation Assay Biochem Kit (Luminometry Based)

Cat # BK121

ORDERING INFORMATION

To order by phone: (303) - 322 - 2254
To order by Fax: (303) - 322 - 2257
Technical assistance: (303) - 322 - 2254
World Wide Web: www.cytoskeleton.com
Email address: cserve@cytoskeleton.com
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Section I: Introduction

Background

The RhoA family of small GTPases consists of at least 20 members, the most extensively characterized of which are the Rac1, RhoA and Cdc42 proteins (1-4). In common with all other small GTPases, the RhoA proteins act as molecular switches that transmit cellular signals through an array of effector proteins. This family mediates a wide range of cellular responses, including cytoskeletal reorganization (1-4), regulation of transcription (5), DNA synthesis, membrane trafficking and apoptosis (6-9).

The RhoA switch operates by alternating between an active, GTP-bound state and an inactive, GDP-bound state (10-12). Understanding the mechanisms that regulate activation / inactivation of the GTPases is of obvious biological significance and is a subject of intense investigation. The fact that many RhoA family effector proteins will specifically recognize the GTP bound form of the protein (13) has been exploited experimentally to develop a powerful affinity purification assay that monitors Rho protein activation (14-16).

Traditionally, this assay has been performed using a pull-down method, wherein a Rho-GTP- binding domain (RBD) of a Rho effector is coupled to agarose beads to affinity purify the active Rho in a biological sample. This method suffers from several drawbacks such as being time consuming, requiring large amount of cell sample, 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 pending) you can now measure Rho activation from cell and tissue samples in less than 4 hours. 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 pulldown	G-LISA
Assay Time	10-12 hours (2 days)	<4 hours
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.

** Quantitative data is obtained by comparing the samples signal to a known concentration of positive control protein.

Outline of the Assay

The RhoA G-LISA™ kit contains a 96 well plate covalently linked to a Rho-GTP-binding protein. Active, GTP-bound Rho in cell lysates will bind to the wells, 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 cells versus non-activated cell lysates. Non-activated RhoA is generally achieved in tissue culture by a serum starvation step (see method). A basic schematic of the steps involved in the G-LISA is shown in Figure 1, representative G-LISA results are shown in Figure 2.

Figure 1: Simple and Quick Protocol

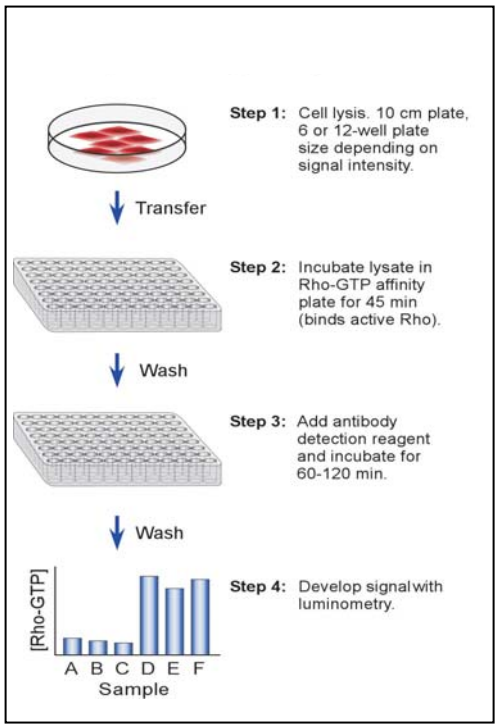
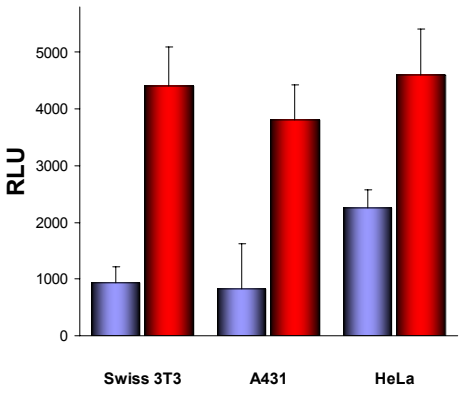


Figure 2: Typical G-LISA Results



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 minutes). 25 µg of cell lysates were subjected to the G-LISA assay. Data

Available Assay Formats

G-LISA assays are available in chemiluminescence and absorbance based formats. Chemiluminescence detection is used for RhoA G-LISA Activation Assay (Cat. # BK121) because the antigen / primary antibody combination produces a low signal. The RhoA,B,C G-LISA Activation Assays (Cat. # BK120 and BK123) are available in chemiluminescence and colorimetric formats because this antigen / primary antibody combination has a large signal. Comparisons of the two assay formats for G-LISA Rho activations are given in Table 2.

Table 2: Comparison of chemiluminescence and absorbance G-LISA assays

	Chemiluminescence	Colorimetric
Assay Time	<4 hours	<4 hours
Cell material per assay	10-50 µg protein (12-well plate)	10-50 µg protein (12-well plate)
Measurement parameters	High gain plus 100ms read per well	405 nm
*Detection Limit**	0.25ng RhoA,B,C for BK120 0.25ng RhoA for BK121	0.50ng RhoA,B,C for BK123
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.

Section II: Important Technical Notes

This section contains detailed notes on the assay, it is present in the full version of the Manual that is shipped with the kit.

Section III. Kit Contents

This kit contains enough reagents for 96 assays. You can run 2 to 96 samples at a time for your own convenience. Table 3 summarizes kit contents.

Table 3: Kit Contents

Reagents	Cat. # or Part # **	Quantity	Storage
96 well Rho-GTP binding domain plate	Part # GL20	96 assays	Desiccated 4°C or room temperature
Plate Strip removal tool	Part # GL21	Enough for 96 assays plus 20% extra.	Room Temperature
Anti-RhoA antibody	Part # GL04	Enough for 96 assays plus 20% extra.	4°C
Secondary antibody - horse radish peroxidase conjugate (HRP)	Part # GL05	Enough for 96 assays plus 20% extra.	4°C
Rho control protein (constitutively active RhoA)	Part # RHCA Similar to Cat# R6301	Enough for 96 assays plus 20% extra.	4°C
Cell lysis buffer	Part # GL36	Enough for 96 assays plus 20% extra.	4°C
Binding buffer	Part # GL37	Enough for 96 assays plus 20% extra.	4°C
Wash buffer	Part # GL38	Enough for 96 assays plus 20% extra.	Room temperature
Antigen presenting buffer	Part # GL39	Enough for 96 assays plus 20% extra.	Room temperature
Antibody dilution buffer	Part # GL40	Enough for 96 assays plus 20% extra.	4°C
HRP detection reagent A	Part # GL41	Enough for 96 assays plus 20% extra.	4°C
HRP detection reagent B	Part # GL42	Enough for 96 assays plus 20% extra.	4°C
Precision Red™ advanced protein assay	Cat. # ADV02 (available as 500 ml size)	Enough for 96 assays plus 20% extra.	Room temperature
Protease inhibitor cocktail	Cat. # PIC02	Enough for 96 assays plus 20% extra.	4°C

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

Section IV. Things to do Prior to Beginning the Assay

This section contains detailed notes on the assay, it is present in the full version of the Manual that is shipped with the kit.

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

Cold 4ºC PBS buffer (10 mM phosphate buffer pH 7.4, 140 mM NaCl, 3 mM KCl)
Multi-channel or multi-dispensing pipettor for 25-200ul range.
Multi-channel pipettor reservoirs
Two microplate shakers, one at room temperature and one at 4^{o}C
Microplate luminometer or other HRP detection system

Section V. Assay Protocol

This section contains detailed notes on the assay, it is present in the full version of the Manual that is shipped with the kit. Here we describe an abbreviated protocol:

Section B: G-LISA™ Protocol for the RhoA Activation Assay.

1. Prepare your cells with respect to your experimental design.
2. Remove the plate in desiccated pouch from storage and dissolve the powder in the wells with 100 µl ice cold nanopure water and keep the plate on ice.
3. Remove excess water from the wells by turning the plate upside down, flick into a waste bin.
4. Add 25 µl of cell extract (0.4mg/ml or greater) or control solution to duplicate wells.
5. Pipette 25 µl binding buffer to all wells and immediately place the plate on a microplate shaker, incubate for 30min.
6. Wash wells with Wash buffer using a multi-channel pipettor.
7. Pipette 200 µl Antigen Presenting Buffer (APB) into each well and incubate at room temperature for 2 min.
8. Flick out the APB and wash the wells once with 200µl of wash buffer.
9. 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.

10. Add 50 µl of diluted anti-RhoA primary antibody to each well and leave the plate on the microplate shaker at room temperature for 45 min.

11. Flick out the Anti-Rho primary antibody and immediately wash the wells with 200µl of wash buffer.

12. 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.

13. Add 50 µl of diluted Secondary antibody to each well and leave the plate on the microplate shaker at room temperature for 45 min.

14. Flick out the Secondary antibody and immediately wash the wells with 200µl of wash buffer.

15. Pipette 50 µl of HRP detection reagent into each well and detect the luminescence signal using a microplate reader.

Section VI. References

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Section VII. Related Products

ITEM CAT#	GTPases	Ras subfamily	Quantity
BK120	G-LISA™ Rho A,B,C Activation Assay (Luminescence based)	Rho	96 assays
BK123	G-LISA™ for Rho A,B,C Activation Assay (Colorimetric based)	Rho	96 assays
BK008	Ras Activation Assay Biochem Kit (Pull down assay)	Ras	25 assays
BK034	Cdc42 Activation Assay Biochem Kit (Pull down assay)	Rho	25 assays
BK035	Rac1 Activation Assay Biochem Kit (Pull down assay)	Rho	25 assays
BK036	Ras Activation Assay Biochem Kit (Pull down assay)	Rho	25 assays
BK100	RhoGEF exchange assay (Fluorimetric)	Rho/Ras	30-100 assays
BK105	Rho GAP assay (Colorimetric)	Rho/Ras	30-100 assays
GE01-A GE01-C	Human Dbs protein (His-tagged)	RhoGEF family	1 x 50 µg 3 x 50 µg
RT02-A RT02-B	Rhotekin-RBD beads	Rho effectors	2 x 500 µg 10 x 500 µg
PAK02-A PAK02-B	PAK-PBD beads	Rho effectors	1 x 250 µg 4 x 250 µg
R6301-A R6301-C	His tagged RhoA (63L) protein	Rho	1 x 20 µg 4 x 20 µg
ADV02	Protein quantitation assay kit	All protein	1 x 500 ml

Section VIII. Trouble-shooting

This section contains detailed notes on the assay, it is present in the full version of the Manual that is shipped with the kit.

Appendix 1: Observation of Actin Morphology By TRITC-phalloidin staining

Reagents needed
Suitable growth media
Bovine serum albumin
LPA stock solution (5 mg/ml in PBS)
PBS solution (150 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2PO_4, 1.47 mM KH_2PO_4)
TRITC-Phalloidin stock (14 mM in methanol, Cat. # PHDR01)
Paraformaldehyde stock (6% stock in PBS, stored aliquoted at -20*C)

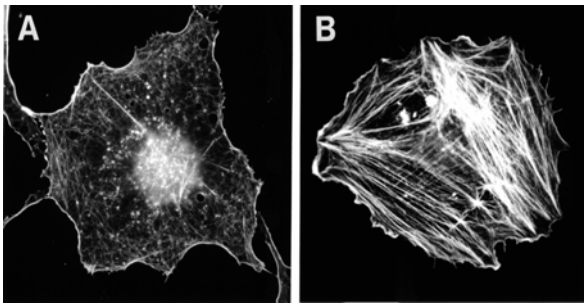
Method
Serum starvation and addition of growth factors

1) Cells are seeded at a density of 3 – 5 x 10^5 cells on an 8.5 cm diameter plate containing four 13 mm diameter glass coverslips.
2) Once cells reach 60% confluence they are washed once in serum free media and then incubated in fresh serum free media for 16 - 20 hours to obtain serum free cultures.
3) After serum starvation remove one coverslip and process for actin staining as described below.
4) Add fresh LPA to the remaining cells to 1 ug/ml and remove a coverslip at times 1 minute, 5 minutes and 20 minutes.
5) Process these for actin staining as described below.

Actin Staining

1) Wash cells once with PBS and fix for 20 minutes at room temperature in 3% paraformaldehyde diluted in PBS.
2) Cells are washed extensively with PBS to remove excess fixative.
3) Treat cells with 0.2% triton in PBS for 5 minutes at room temperature to permeabilize cells.
4) Wash twice in PBS.
5) Incubate with 200 ul of 0.1 ug/ml TRITC-phalloidin for 30 minutes at room temperature in the dark.
6) Wash five times with PBS.
7) Mount cells in mounting medium and allow to set for 60 minutes.
8) View actin filaments with a 63 – 100 X oil immersion objective.
9) Examples of serum starved and LPA treated cells are shown in Fig. 1.

Figure 1: Phalloidin Staining of the Actin Cytoskeleton in Serum Starved and LPA Treated Cells



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 TRITC-phalloidin. B) Cells treated for 5 minutes with 1 ug/ml LPA after serum starvation and subsequently stained with TRITC-phalloidin.

Appendix 2: Known Rho protein activators

Activator*	Treatment	Cell line used	Response	Type of Assay Used	Reference
Lysophospatidic acid (LPA) *(serum lipid & G-coupled receptor agonist)*	1 ug/ml	Swiss 3T3 cells, adherent & suspension	Maximal activation of 2-6 fold after 1 minute then dropping to basal after 30 minutes	Rhotekin-RBD pulldown	20
Lysophospatidic acid (LPA) *(serum lipid & G-coupled receptor agonist)*	1 uM	N1E-115 neuronal cells	Maximal activation of 3-5 fold after 3 minutes	Rho-kinase pull down assay	22
Serum	5 - 10%	Swiss 3T3 cells, adherent or suspension	Maximal activation of 2-6 fold (10%) & 1-2 fold (5%) after 1-5 minutes	Rhotekin-RBD pulldown	20
Fibronectin *(extracellular matrix protein)*	Culture plate is coated with fibronectin	Swiss 3T3 cells	Biphasic regulation after plating cells on fibronection coated plates. Initial period of low RhoA activity (10-20 minutes) followed by a 1-7 fold activation peaking at 60-90 minutes and then dropping to basal levels after 6 hrs.	Rhotekin-RBD pulldown	20
Calpeptin *(protease)*	100ug/ml	Swiss 3T3 cells adherent	Maximal activation after 5 to 10min with extended activation time up to 30min, decreasing thereafter to basal levels after 60min.		
Thrombin *(protease)*	10 nM	HUVEC human venous endothelial primary cells (fifth passage cells)	Maximal activation of 14 fold after 2 minutes, dropping to basal levels after 30 minutes	Rhotekin-RBD pulldown	19
Colchicine *(microtubule destabilizer)*	10 ug/ml	Swiss 3T3 cells, adherent or suspension	Maximal activation of 2-4 fold activation after 30 minutes	Rhotekin-RBD pulldown	20
Nocodazole *(microtubule destabilizer)*	10 uM	MG63 human osteosarcoma cells & HeLa cells	Maximal activation of 2-3 fold activation after 30 minutes	Actin morphology, Rhotekin-RBD pulldown	21, 23
Vinblastine *(microtubule destabilizer)*	50 uM	MG63 human osteosarcoma cells	Maximal activation of 2-4 fold activation after 30 minutes	Actin morphology	21
Cytochalasin D *(actin filament destabilizer)*	0.5 ug/ml	Swiss 3T3 cells, adherent or suspension	Maximal activation of 1-2 fold after 60 minutes	Rhotekin-RBD pulldown	20
Spingosine -1-phosphate *(serum lipid & G-coupled receptor agonist)*	1 ug/ml	Swiss 3T3 cells, adherent or suspension	Maximal activation of 1-2 fold after 2 minutes for 3T3 cells and 20 minutes for HUVEC cells	Rhotekin-RBD pulldown	20, 19
Bombesin	10 nM	Swiss 3T3 cells	Maximal activation of 2-3 fold after 1 minute which is sustained for at least 30 minutes	Actin morphology	24

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