Manual

V 6.1

G-LISA® Ral Activation Assay

Biochem Kit™

(Colorimetric Based)

Cat. # BK129

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Manual Contents

I: Introduction

Background

The Ras family of small GTPases consists of at least 35 related human proteins that include the oncoproteins HRAS, KRAS and NRAS as the founding members (Colicelli 2004). The Ral proteins, RalA and RalB, share 58% identity to these oncoproteins and 85% identity with each other (Chardin and Tavitian 1986). Ral proteins play an important role in diverse cellular processes including endocytosis, exocytosis, oncogenesis and the regulation of transcription and cell morphology (Feig 2003)

Like other small GTPases, Ral proteins become activated when they switch from the GDP -bound state to the GTP-bound state (Takai, Sasaki and Matozaki 2001), and it is the GTP-bound form that specifically interacts with their downstream effector proteins. Traditionally, Ral activation has been evaluated using a pull-down assay method, wherein the Ral-GTP binding domain (RBD) of a Ral effector is coupled to glutathione beads, allowing affinity based detection of the active Ral in biological samples (*e.g* (Hofer, Berdeaux and Martin 1998)). This method suffers from several drawbacks such as being time consuming, requiring large amounts of total cellular protein, being limited in the number of samples that can be handled simultaneously and yielding only semi-quantitative results.

The Ral G-LISA® Advantage

With the new G-LISA® kit (patent pending) you can now measure Ral activation from cell and tissue samples in less than 2 h. G-LISA® requires only 1-5% of the material needed for a conventional pull-down assay. You will also be able to handle large sample numbers and generate quantitative results. The G-LISA® advantages are summarized in Table 1.

Table 1: The G-LISA® Advantage

	Traditional pull-down	G-LISA®
Assay Time	10-12 h (2 days)	<3 h
Cell material per assay	0.5-2 mg protein (100 mm plate)	5-25 µg protein (12-well plate)
*Lysate clarification needed**	Yes	No
Sample handling	Up to 10 samples	Up to 96 samples (or more)
*Quantitative Data***	Semi	Yes
High throughput compatible	No	Yes

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

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

Assay Principle

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

Figure 1: Simple and Quick Protocol

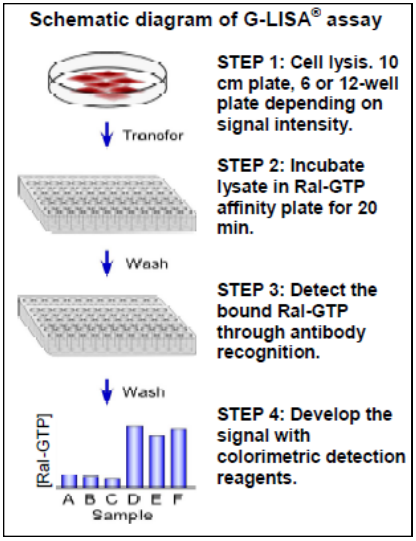


Figure 2: Typical G-LISA® Results

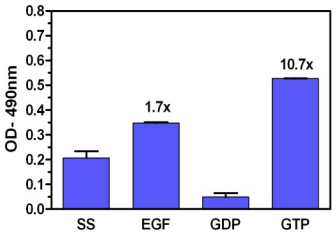


Figure 2. *Ral activation measured using the Ral G-LISA® format. Serum starved Rat-2 cell extract and EGF activated Rat-2 cell extract were incubated in separate wells of the G-LISA® plate. Extracts from Rat-2 cells were also loaded with either GDP or GTP to investigate the maximal Ral activation window available in Rat-2 cells. The wells were probed with anti-Ral monoclonal antibody (Part# GL08) and a secondary antibody. Finally, the plate was developed with a colorimetric substrate and the absorbance was read at 490 nm.*

II: Purchaser Notification

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. 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 can not sell or otherwise transfer this product or any component thereof to a third party or otherwise use this product or its components for commercial purposes. Commercial purposes include, but are not limited to: use of the product or its components in manufacturing; use of the product or its components to provide a service; resale of the product or its components.

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

III: Kit Contents

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

Table 2: Kit Contents

Reagents	Cat. # or Part # *	Quantity	Storage
96 well Ral-GTP binding plate	Part # GL34	12 strips of 8 wells each	Desiccated 4°C Stable for 6 months
Anti-RalA monoclonal antibody	Part # GL08	1 tube, lyophilized	Desiccated 4°C Stable for 6 months
Secondary antibody – horseradish peroxidase conjugate (HRP)	Part # GL02	1 tube, lyophilized	Desiccated 4°C Stable for 6 months
RalA control protein (constitutively active RalA)	Part # RLCA	12 tubes, lyophilized	Desiccated 4°C Stable for 6 months
Ral Binding Buffer	Part # GL52	1 bottle, lyophilized	4°C Stable for 6 months
Cell Lysis Buffer	Part # GL36	1 bottle, lyophilized	Desiccated 4°C Stable for 6 months
Wash Buffer	Part # GL38	1 bottle, lyophilized	Desiccated 4°C Stable for 6 months
Antigen Presenting Buffer	Part # GL39	1 bottle, 25 ml	Room temperature Stable for 6 months
Antibody Dilution Buffer	Part # GL40	1 bottle, lyophilized	Desiccated 4°C Stable for 6 months
HRP Detection Reagent A	Part # GL43	1 tablet, silver pack	4°C Stable for 6 months
HRP Detection Reagent B	Part # GL44	1 tablet, gold pack	4°C Stable for 6 months
HRP Reagent Stop Solution	Part # GL80	2 bottles, 8 ml each	4°C Stable for 6 months
Precision Red™ Advanced Protein Assay	Part # GL50 (available in 500 ml size Cat. # ADV02)	1 bottle, 100 ml	Room temperature Stable for 6 months
Protease Inhibitor Cocktail	Cat. # PIC02	1 tube, lyophilized	Desiccated 4°C Stable for 6 months

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

The reagents and equipment that are required but not supplied:

- Cold 4°C PBS buffer (10 mM phosphate buffer pH 7.4, 140 mM NaCl, 3 mM KCl).
- Cell scrapers
- 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.
- Two orbital microplate shakers (VWR, Cat. # 57019). Optimal shaker speed is 400 rpm (200 rpm is the minimal speed required). One at room temperature and one at 4°C.
- Microplate reader (see Section V: Important Technical Notes for information on settings etc.)

IV: Reconstitution and Storage of Components

Many of the components of this kit have been provided in lyophilized form. Prior to beginning the assay you will need to reconstitute several components as shown in Table 3:

Table 3: Component Storage and Reconstitution

Kit Component	Reconstitution	Storage Conditions
Ral-GTP binding 96 well plate	It is imperative to keep the plate in the sealed desiccant bag with desiccant at all times. Reconstitution is not necessary prior to the start of the assay. The protective white powder pellet in each well of the plate may become detached from the bottom of the well during shipping. This will not affect the assay performance. Pellets should be tapped to the bottom of the well prior to resuspension.	Store desiccated at 4°C or room temperature Stable for 6 months
Anti-RalA antibody	Resuspend one vial in 200 µl of PBS with 0.1% sodium azide and pipette up and down three times	Store at 4°C Stable for 6 months
Secondary antibody HRP	Centrifuge briefly to collect the pellet in the bottom of the tube. Dissolve the powder in 80 µl of PBS. Do not use sodium azide in combination with this antibody as it will inactivate the HRP.	Store at 4°C Stable for 6 months
RalA control protein (12 tubes)	Resuspend one vial in 500 µl of Cell Lysis Buffer and pipette up and down three times.	Store at 4°C Stable for 6 months
Cell Lysis Buffer	Reconstitute in 100 ml of sterile distilled water. This may take 5-10 min to resuspend. Use a 10 ml pipette to thoroughly resuspend the buffer.	Store at 4°C Stable for 1 year
Binding Buffer	Resuspend in 10 ml of distilled water	Store at 4°C Stable for 6 months
Wash Buffer	Reconstitute in 1000 ml of distilled water. This will take 45-60 min to resuspend. A magnetic stir bar and stir plate can be used to help resuspension.	Store at room temperature Stable for 6 months
Antigen Presenting Buffer	No reconstitution necessary.	Store at room temperature Stable for 6 months
Antibody Dilution Buffer	Reconstitute in 15 ml of sterile Milli-Q water.	Store at 4°C Stable for 6 months
HRP Detection Reagent A	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 12 x 0.8 ml volumes. Place in -70°C freezer for storage. NOTE -20°C is NOT good for storage.	Store at -70°C Stable for 6 months
HRP Detection reagent B	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 12 x 0.8 ml volumes. Place in -70°C freezer for storage. NOTE -20°C is NOT good for storage.	Store at -70°C Stable for 6 months
Stop Buffer	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. Mix well and store at 4°C.	Store at 4°C Stable for 6 months
Precision Red™ Advanced Protein Assay	No reconstitution necessary.	Store at room temperature Stable for 6 months
Protease Inhibitor Cocktail	Reconstitute in 1 ml of dimethyl sulfoxide (DMSO) for 100 x stock.	Store at 4°C Stable for 6 month

V: Important Technical Notes

A. Update to Version 6.0 and up

Part# ARL01 has been updated to part#GL08. The production of this Antibody has been further optimized for this kit.

B. Growth and Treatment of Cell Lines

The health and responsiveness of your cell line is the single most important parameter for the success and reproducibility of Ral activation assays. The next most important parameters are time course of activation and titration of the activating factor (see Section V, subsection D). Time should be taken to read this section and to carefully maintain cell lines in accordance with the guidelines given below.

Adherent cells should be ready when they are 50-70% confluent. Non-adherent cells should be ready at approximately 3×10^5 cells per ml. Briefly, cells are seeded at 5×10^4 per ml and grow for 3 days. Serum starvation or other treatment will be performed when they are 50% confluent (see Appendix 1).

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

Cells should also be checked for their responsiveness ("responsive state") to a known stimulus for example EGF, LPA or ionomycin. In many cases poor culturing technique can result in essentially non-responsive cells. An example of poor culturing technique includes the sub-culture of cells that have previously been allowed to become overgrown. In general, cells grown to >80% confluence should not be used for Ral activation studies.

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

C. Assay Preparation For G-LISA®

It is critical to get the assay components ready before preparing cell lysates or thawing previously prepared lysates, as a defrosted activated lysate is unstable and should be assayed as soon as possible after preparation.

Table 4: Assay Preparation for G-LISA®

Reagent	Preparation
Ral-GTP binding 96 well plate	1. Remove plate from 4°C and keep in its protective bag, place on your bench at room temperature for 30 min. 2. Do not remove the plate (or strips) from the bag until immediately prior to the experiment.
Distilled water	30 ml in a 50ml Falcon tube on ice.
Protease Inhibitor Cocktail	Keep at room temperature.
Lysis Buffer	Either pipette 10ml of Lysis buffer into 15ml Falcon and pipette 100µl of PIC02 into it, mix and place in ice, or, 1. Or determine volume of Lysis Buffer needed per culture vessel by looking at Table 5. 2. Determine **total volume** of Lysis Buffer needed by multiplying the lysis volume per culture vessel (µl) by number of vessels x 1.3 (see Table 5 for guidelines). 3. Aliquot this volume of Lysis Buffer into a clearly labeled tube and place in ice. 4. Add 10 µl of protease inhibitor cocktail per ml of aliquoted Lysis Buffer. 5. Mix well and leave on ice. 6. Lysis Buffer needs to be **ice cold**.
Binding Buffer	Place reconstituted buffer on ice.
RalA control protein	This can be removed from 4°C storage and placed on ice.
Anti-RalA antibody	Have primary antibody stock ready on ice. For two 8-well strips, you will need to mix 10 µl antibody with 500 µl Antibody Dilution Buffer (1/50 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.
Precision Red™ Advanced Protein Assay Reagent	Place on the bench and use at room temperature.

D. Timing and Titration of Activator

Upon stimulation, Ral proteins are generally activated very rapidly and transiently. Maximal activation ranges from 30 s to 30 min and declines thereafter to basal levels. For potent activators such as EGF or LPA, the intensity of maximal Ral activation over "control state" (serum starved) cells is generally in the order of 2-5 fold (Hofer, Berdeaux and Martin 1998). However using a single time point you are more likely to miss this maximum activation peak. It is therefore critical to take timed samples for at least the first experiment with an unknown activating entity. Recommended time points are 0, 1, 3, 6, 12 and 30 min, which fit nicely into a 6 well culture plate (The time course is also recommended for Ral inactivation studies).

In practical terms the timed experiment should be performed sequentially. This allows rapid processing of each single time point. Once one time point lysate is collected, it should be snap frozen in "experiment sized" aliquots immediately and kept in -70°C. The G-LISA® kit uses 25 µl of lysate (0.25-1 mg/ml lysate protein concentration) per assay. We recommend duplicate or triplicate samples per timepoint or condition, therefore 60-100 µl aliquots are recommended for snap freezing.

E. Rapid processing of cells

GTP bound (active) Ral is a labile entity, the bound GTP is susceptible to hydrolysis by Ral-GAPs during and after cell lysis resulting in Ral inactivation. Also GEF and GAP activities in extracts contribute to a change in the actual state of activation prior to lysis. Rapid processing at 4°C is essential for accurate and reproducible results. The following guidelines are useful for rapid washing of cells.

Washing

1. Retrieve culture dish from incubator, immediately aspirate out all of the media.
2. Immediately rinse cells with an appropriate volume of ICE COLD PBS to remove serum proteins (see Table 5 for recommended wash volumes) and place the plate on ice.
3. Aspirate off all residual PBS buffer. This is essential so that the Lysis Buffer is not diluted. Correct aspiration requires that the culture dish be placed at an angle on ice for 10 to 30 s to allow excess PBS to collect in the vessel for complete removal. Do not leave on ice too long because the cells may respond to this temperature change.

Cell Lysis

To avoid making too dilute or too concentrated lysate samples (<0.15 or >1.0 mg/ml), it is recommended to adjust the amount of Lysis Buffer depending on your cell type and plate type. Table 5 gives guidelines for suitable lysis volumes for 3T3 cells which tend to give low protein yields. The exact lysis volumes for any given cell line will have to be determined empirically.

Table 5: Recommended Wash and Lysis Volumes for 3T3 Cell Cultures

Culture Vessel	Vessel surface area (cm^2)	Volume of PBS wash (ml)	Volume of Lysis Buffer (µl)
35 mm dish	8	2.0	100
60 mm dish	21	3.0	250
100 mm dish	56	10.0	600
150 mm dish	148	15.0	2000
6-well cluster plate	9.5 / well	3.0	100
12-well cluster plate	4 / well	1.5	60
T-25 Flask	25	4.0	250
T-75 Flask	75	10.0	1200
T-150 Flask	150	15.0	2000

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

1. Work quickly.
2. Keep solutions and lysates embedded in ice so that the temperature is below 4°C. This helps to minimize changes in signal over time. The Assay Protocol (Section VI) gives very specific instructions regarding temperature and must be strictly adhered to for successful results.
3. We strongly recommend cell lysates should be immediately frozen after harvest and clarification. A sample of at least 20 µl should be kept on ice for protein concentration measurement. The lysates must be snap frozen in liquid nitrogen and stored at -70°C. Lysates can be stored at -70°C for up to 3 months.
4. Thawing of cell lysates prior to the use in the G-LISA® assay should be in a room temperature water bath until 30% ice is left, followed by rapid transfer to ice and immediate use in the assay. Do not leave the extracts in the water bath to thaw completely, the remaining frozen lysate will thaw quickly once transferred to ice.

F. Protein Concentration:

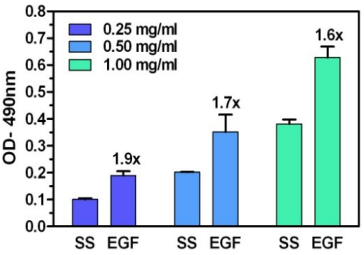
Equal protein concentration in all samples is a prerequisite for accurate comparison between samples in Ral activation assays. Cell extracts should be equalized with ice cold buffer plus protease inhibitor cocktail. We recommend 0.5 mg/ml total protein as a good starting point. For example, cell lysates of protein concentrations ranging from 0.6–1.3 mg/ml would all need to be diluted to 0.5 mg/ml. However, due to the difference of Rac-GTP levels in different cell lines, we highly recommend you titrate your lysate. Serially dilute your lysate from “controlled cell state” samples (e.g. serum starved) and perform a G-LISA® assay with them. Pick the lysate concentration that gives OD reading (after buffer blank subtraction) between 0.2-0.4. By doing this the OD readings of activated cell vs. controlled cell are more likely to stay in the linear range of the assay.

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

G. Assay Linearity

The assay is linear from 0.5 ng to 5 ng of bound activated Ral. The positive control protein is at 2.5 ng, therefore if any OD readings are more than 2 times this sample they will be out of the linear range of the assay. In such cases you should reduce the amount of total cell protein per assay. The lower level of detection of the assay is approximately 20% above the background reading of Lysis Buffer only. Readings lower than this will require increased cell protein per assay. A typical linearity test of the lysate concentration is shown in Figure 3.

Figure 3. Ral activation by EGF measured by G-LISA®. Rat-2 cells were serum starved (SS) for 24 h and treated with EGF (100 ng/ml for 2 min). Lysates from these cells were tested at 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml starting concentrations in the Ral G-LISA® assay. Absorbance was measured at 490 nm.



H. Use of a Multi-channel Pipettor

When processing more than 16 wells it is imperative to use a multi-channel or multi-dispensing pipettor with a range of 25 to 200 µl per dispense. Critical steps such as lysate addition, post-binding wash step and the Antigen Presenting Buffer step all have requirements for accurate and timely additions. Attempting to perform >16 assays with a single channel pipettor will also increase the likelihood of allowing wells to dry out before reagent addition can be completed, resulting in variable signals. Therefore, use a multi-channel or at least a multi-dispensing pipettor wherever possible. If neither of these pipettor options is available we highly recommend that you limit each experiment to a maximum of 16 wells.

I. Plate Shaker Recommendations

It is essential to use an orbital plate shaker at 400 rpm. Rocking or tilting plate shakers will not be sufficient for this assay. As a back-up you can use a 200 rpm orbital shaking culture incubator or a normal orbital rotating platform. Signals will be lower with the 200 rpm option. Here are a few suppliers with suitable orbital plate shakers:

Model # 4625 Titer Plate Shaker, Lab-Line Instruments, Barnstead Intl.
(average price)

Model # RF7854 Digital Microplate Shaker, ML Market Lab, researchml.com
(economical price)

Model # RF7855 Incubating Microplate Shaker, ML Market Lab, researchml.com
(deluxe model)

J. Plate Reader Settings

Table 6. Plate reader settings

Parameters	Character	Contents
Wavelength	490 nm	Bandwidth 20nm or less
Shake	Orbital, normal rate	5 s
Temperature	Room temperature	
Protocol Type	End point	

VI: Assay Protocol

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

STEP 1: Assay Preparation

At least one hour prior to beginning the assay, prepare all G-LISA® assay components as described in Section IV and Section V: Important Technical Notes, Table 4. Use the check -off list below to confirm that the following reagents are ready;

- □ Ral-GTP binding plate, at room temperature in the desiccant bag
- □ Wash Buffer, resuspended at room temperature
- □ Precision Red™ Advanced Protein Assay Reagent, room temperature
- □ Distilled water, 30 ml, ice cold
- □ Lysis Buffer, ice cold with protease inhibitors (see Table 5)
- □ PBS, ice cold, if necessary (see Section V (E) and Table 5)
- □ Labeled 1.5 ml microcentrifuge tubes, on ice.
- □ Ral Control Protein, resuspended in Resuspension Buffer (500 µl per tube) on ice
- □ Antibody Dilution Buffer, room temperature

STEP 2: Lysate collection

We strongly recommend you snap freeze your cell lysate in liquid nitrogen right after you harvest and clarify. This is especially necessary if you have many samples. Save a small amount of lysate before you snap freeze them for protein assay.

1. Treat cells as your experiment requires.
2. After treatment, aspirate media and if necessary wash with ice cold PBS (see Table 5 of Section V (E) for recommended volumes and additional discussion of the washing step).
3. Aspirate off PBS. Tilt plates on ice for an additional 10 to 30 s and re-aspirate all remnants of PBS. Residual PBS will adversely affect the assay.
4. Pipette the appropriate volume of ice-cold Cell Lysis Buffer directly onto cells and place on ice (see Table 5 of Section V (E) for recommended volumes).
5. While the plate is still on ice, disrupt cells with a cell scraper and pipette the extract from the bottom corner of the tilted plate into a pre-labeled sample tube on ice. At this point each lysate volume should not exceed 130% of the original Lysis Buffer volume.
6. Immediately clarify by centrifugation at 6,000 xg (9,000 rpm in microfuge), 4°C for 2 min.
7. Save 60 µl of each lysate aside in a microcentrifuge tube for the protein assay.
8. Aliquot the remaining cell lysates into the pre-labeled tubes and snap freeze liquid nitrogen. Keep them at -70°C for future use. It is recommended to aliquot 120 µl volumes per tube.

9. Measure lysate protein concentration as follows:

   A. Add 20 µl of each lysate or Lysis Buffer into disposable 1 ml cuvettes.
   B. Add 1 ml of Protein Assay Reagent (Part # GL50) to each cuvette.
   C. Incubate for 1 min at room temperature.
   D. Blank spectrophotometer with the Lysis Buffer + Protein Assay Reagent cuvette at 600 nm.
   E. Read absorbance of samples.
   F. Multiply the absorbance by 5 to obtain the protein concentration in mg/ml.

10. When all the samples have been processed then move on to the next step.
11. Calculate the volume required to equalize the cell extracts with ice cold Lysis Buffer plus protease inhibitors. We recommend equalization to approximately 0.5 mg/ml as a good starting point for this assay. It is essential to have equal protein concentration in each sample to effectively compare samples. Other protein concentrations can be chosen e.g. 1.00 mg/ml, based on empirical observation of the signals with your particular extracts or based on the lowest concentration sample being lower than 0.25 mg/ml in this case the lowest protein concentration would be chosen for equalization. Specifically, we recommend a final concentration of 0.5 mg/ml for Swiss 3T3 cell lysates.

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

If using the 1ml protein assay, then use this formula:

$$\frac{A - B}{B} \times (\text{volume of A, usually 60ul}) = \_\_\_\_\_ \ \mu l \text{ of Lysis Buffer plus protease inhibitors}$$

Where A is the protein concentration of each extract and B is the chosen protein concentration for equalization, usually 0.25 mg/ml.

STEP 3. G-LISA® assay

1. Aliquot 60 µl of Lysis Buffer into a labeled microfuge tube and dilute with 60 µl of cold Binding Buffer. Keep on ice. This is volume is sufficient for two wells of buffer blank control.
2. Aliquot 30 µl of RLCA (Ral Control Protein) and 30 µl of Lysis Buffer into a labeled microfuge tube and dilute with 60 µl of cold Binding Buffer. Keep on ice. This volume is sufficient for two wells of positive control.
3. Take the Ral-GTP binding plate out of its bag. Gently peel up the seal from the strips and pull out the number of strips required. Place strips in the extra strip holder provided, and place on ice. Immediately after removing the strips needed, put the rest of the plate back in the pouch with desiccant and place back in storage.
4. Dissolve the powder in the wells with 100 µl ice cold water.
5. Thaw the snap frozen cell lysates in ROOM TEMPERATURE water bath. Immediately place on ice after they are thawed.
6. Based on the calculation of equalization, add required amount of Lysis Buffer to respective tubes to equalize all lysate concentration.

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

7. Aliquot sufficient lysate for duplicate (60 µl) or triplicate (90 µl) assays into ice cold microcentrifuge tubes.
8. Add an equal volume if ice-cold Binding Buffer to each tube, mix well with a pipette. Keep on ice.
9. Completely remove the water from the microplate wells as follows:

   Complete removal of solutions from the well requires a vigorous flick of the plate and a vigorous series of pats onto paper towels (5-7 hard pats). The complete removal of solution from wells between steps of the G-LISA® is very important as it avoids high background readings in the buffer only wells. The buffer only wells should read between 0.30 – 0.4 at an absorbance of 490 nm. If background readings are higher than 0.4 then a more vigorous removal of solutions from the well should be practiced.
10. Put plate back on ice.
11. Immediately add 50 µl of lysate to respective wells.
12. Pipette 50 µl of buffer blank control to duplicate wells.
13. Pipette 50 µl of Ral positive control into duplicate wells.
14. Immediately place the plate on a cold orbital microplate shaker (400 rpm recommended, 200 rpm minimum) at 4°C for exactly 20 min.

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

15. During the incubation, dilute the anti-Ral primary antibody to 1/50 in Antibody Dilution Buffer by adding 10 µl of antibody to every 500 µl Antibody Dilution Buffer. Note: A volume of 500 µl is required for one strip (8 wells).

16. After 20 min, flick out the solution from the wells and wash twice with 200 µl Wash Buffer at room temperature using a multi-channel pipettor. Do not leave this plate unattended at this time. Vigorously remove the Wash Buffer after each wash by flicking and patting the plate as detailed in step 9.

17. Place plate on the bench.

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

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

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

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

22. During the primary antibody incubation, dilute the secondary HRP labeled antibody to 1/100 in Antibody Dilution Buffer by adding 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).

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

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

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

26. During the secondary antibody incubation prepare the HRP detection reagent. The HRP reagent is in two parts (A & B) which should be mixed in equal volumes prior to adding 50 µl to each well. For each strip of 8 wells, mix 250 µl of Reagent A with 250 µl of Reagent B into a clean tube. Components A and B should be thawed in a room temperature water bath and removed as soon as they are thawed. The mixture must be protected from light and is stable at room temperature for 1 h. Unused mixed solution should be discarded. Unused, non-mixed, solutions should be re-frozen immediately for later use.

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

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

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

30. Add 50 µl of HRP Stop Buffer.

31. Immediately read the signal by measuring absorbance at 490 nm using a microplate spectrophotometer as described in Section V: Important Technical Notes. Designate Lysis Buffer only wells as the assay Blank.

VII: Data Analysis

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

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

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

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

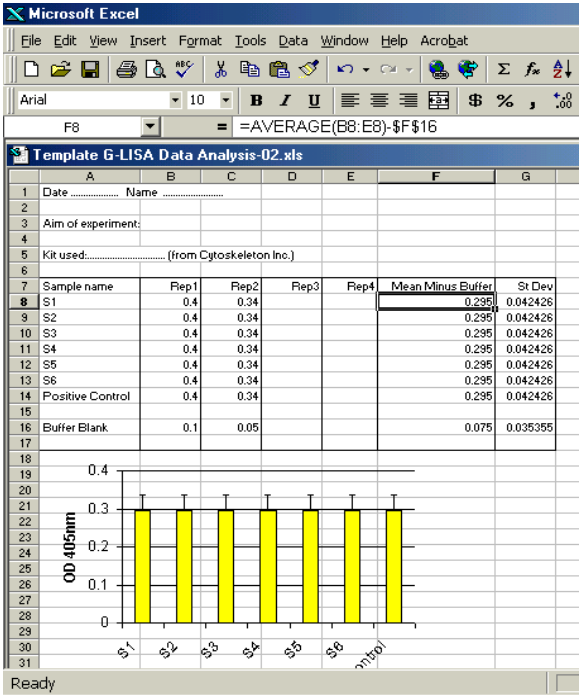
5. Enter the following formula into the first sector under "Standard deviation", "=stdev (Xn:Yn)" where X = the column designator for "rep1", Y = column designator for "rep4", and n= row designator of the row that you are working on. Repeat for each sector under the "Standard deviation" header until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

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

7. When the data has been entered select the Sample, Mean and Standard deviation data sectors by the click and drag method. Then select the chart making function, in Excel this looks like a clickable square with a mini-bar chart in. This will guide you through the chart making process with the data you have selected. Choose "column chart" initially, designate the Mean numbers for input values. The Standard deviation column for the y-axis error bars needs to be designated after the Mean numbers chart is made. This is achieved by double clicking on the graph bars, and selecting the "Y-axis error" tab, then entering the location of the standard deviation data by clicking the "Custom" option and selecting the area in the worksheet. E-mail tservice@cytoskeleton.com for a free Excel Template. An example of a typical Excel layout and data plot is shown in Figures 5.

VII: Data Analysis (Continued)

Figure 5: Typical Excel Layout



VIII: Troubleshooting Guide

Observation	Possible cause	Remedy
Weak signal or no signal in all wells	1. Slow processing of samples or processing at above 4°C 2. The wells were allowed to dry out during the experiment. 3. The plate was allowed to get damp during storage. 4. A step or component of the assay was omitted *e.g.* Stop solution acid not added. 5. HRP color not developed long enough.	1. Process samples quickly on ice. 2. Do not remove the solution in the wells unless the solution of next step is ready. 3. Store the plate in the desiccant bag with the bag securely sealed. Keep the foil cover on the plate. 4. Read instructions carefully. 5. Try 20 or 30min incubation.
High signal in all wells	1. Concentration of antibodies is too high. 2. Insufficient washes were performed.	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 plate washing (G-LISA® assay protocol Step 7).
High signal in positive control relative to samples	1. Incorrect plate shaker 2. Too much positive control or too little protein lysate.	1. Use an orbital shaker described in Section V(J). 2. Check volumes used and compare to the manual.
Induced sample does not give significant signal increase	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-Ral 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 new vial of inducer or titrate the activator as in Fig.3, Section V(G). 2. Read instructions carefully and compare with your Experiment Record Sheet 3. The linear range of the assay is 0.5 ng - 5 ng Ral. 4. Titrate down the amount of extract to be added. 5. Details of how to serum starve cells is given in Appendix 1. 6. Lyse cells on ice, keep all cell lysates, Lysis Buffer, PBS and distilled water on ice. 7. Remove PBS wash which may be causing a cell response that is the same in all samples.
Significant variation between duplicate/ triplicate 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 incorrectly stored after reconstitution.	1. Use a second vial of RLCA.

IX: References

1. Chardin, P., and A. Tavitian. "The ral gene: a new ras-related gene isolated by the use of a synthetic probe." *EMBO J 5* (1986): 2203-2208.

2. Colicelli, J. "Human RAS Superfamily Proteins and Related GTPases." *Sci STKE 250*, no. Re13 (2004): 1-53.

3. Feig, L.A. "Ral-GTPases: approaching their 15 minutes of fame." *Trends in Cell Biology* 13, no. 8 (2003): 419-425.

4. Hofer, F., R. Berdeaux, and G.S. Martin. "Ras-independent activation of Ral by a Ca2+-dependent pathway." *Current Biology* 8 (1998): 839-842.

5. Takai, Y., T. Sasaki, and T. Matozaki. "Small GTP-binding proteins." *Physiol. Rev.* 81 (2001): 153-208.

Appendix 1: Observation of Actin Morphology

Reagents needed

- □ Control state cells and Responsive state cells (e.g. serum starved cells and EGF treated cells).
- □ PBS solution (10 mM phosphate buffer pH 7.4, 140 mM NaCl, 3 mM KCl)
- □ Rhodamine-phalloidin stock (14 mM in methanol, Cat. # PHDR1)
- □ Fixative Solution (4% formaldehyde in PBS)
- □ Permeabilization Buffer (0.5% Triton in PBS)
- □ Antifade Mounting Medium
- □ All above reagents (except cells) are available in a convenient kit format from Cytoskeleton Inc. (Actin Staining Biochem Kit™, Cat. # BK005)

Method

Serum starvation and addition of growth factors

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

Actin Staining

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

Appendix 1: Staining Protocol for F-actin (Cont.)

5. Permeabilize cells by incubating in permeabilization buffer for 5 min at room temperature.

6. Wash once in PBS for 30 s at room temperature.

7. Incubate with working stock rhodamine-phalloidin for 30 min at room temperature in the dark.

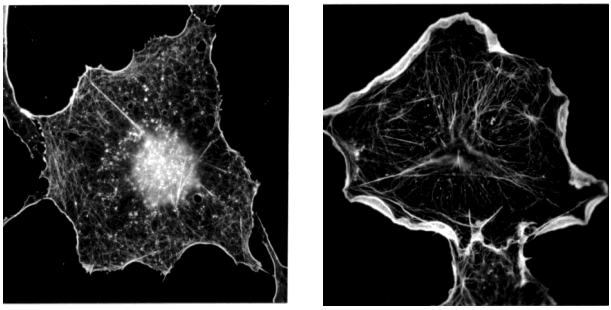
8. Wash three times with PBS.

9. Mount cells in mounting medium and allow to set for 60 min in the dark.

10. View actin filaments by fluorescence microscopy (excitation filter 535 nm, emission filter 585 nm).

11. Examples of serum starved and EGF treated cells are shown in Fig. 5. Characteristic membrane ruffles and lamellipodia should be observed in Rac activated cells.

Figure 5: Rhodamine Phalloidin Staining of the Actin Cytoskeleton in Serum Starved and EGF 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 rhodamine-phalloidin. Left: Serum starved cells untreated; Right: Cells treated for 2 min with 10 ng/ml EGF after serum starvation and subsequently stained with rhodamine phalloidin.

Appendix 2: Experiment Record Sheet

Scientist Name …….

Contact Tel. # …….

e-mail …….

Kit Cat. # / Lot # …….

STEP		Comments or Changes
1	Type of cells or tissue ……..	
2	How were the cells prepared prior to lysis?	……. days in culture
		……...% confluency
		…… inducer
		……...mg/ml of protein in lysate

3. Was a wash step employed prior to lysis? ……Y or N

4. Time that cultures were removed from incubator? ……am or pm

5. Time that reactions were placed on the shaker? ……am or pm

7. Did you add 50 µl of extract per well? ……Y or N

8. What locations are the 50 µl Lysis Buffer controls? ……..…...wells

9. What speed and time was the shaking for the binding reaction?

……..rpm ……..……min

10. How long did you wait after the post-binding wash step? ……..…..s or min

11. What was the time when the anti-Ral primary antibody reaction was started?

….……..am or pm

12. What was the time when the Secondary antibody reaction was started?

……...am or pm

13. What was the time when detection reagent was added? ……am or pm

14. What was the time when the plate was read? ……am or pm

Appendix 4: Plate Record Template

Name of experiment: ……..

Date of experiment: ……