V 4.0

G-LISA® Ras Activation Assay Biochem Kit™ (Absorbance Based)

Cat. # BK131

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

Manual Contents

I: Introduction

Background

The Ras small G-proteins act as molecular switches that transmit cellular signals through an array of effector proteins. The mammalian Ras subfamily of proteins (H-Ras, N-Ras, K -Ras4A and K-Ras4B) play important roles in many cellular functions including the control of cell proliferation, differentiation and cell death (1).

The Ras switch operates by alternating between an active, GTP-bound state and an inactive, GDP-bound state (2-4). Oncogenic mutations that lead to the expression of constitutively active/GTP-bound Ras are found in ~30% of human malignant tumors derived from a diverse range of tissues (5).

The fact that many Ras effector proteins will specifically recognize the GTP bound form of the protein has been exploited experimentally to develop affinity based separation methods that allow for the isolation and detection of activated Ras in biological samples (6-8). These Ras "pull-down" activation assays typically utilize the Ras binding domain (RBD) of a Ras effector protein coupled to agarose beads, which allows the active Ras to be separated from the bulk of the biological sample by virtue of its interaction with the RBD. The sample is then analyzed by Western blot to determine the levels of active Ras that were bound to the beads. This method suffers from several drawbacks, however, 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 Ras G-LISA® Advantage

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

Table 1: The G-LISA® Advantage

	Traditional pull-down	G-LISA®
Assay Time	10-12 h (2 days)	<3 h
Cell material per assay	0.5-2 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

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

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

Assay Principle

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

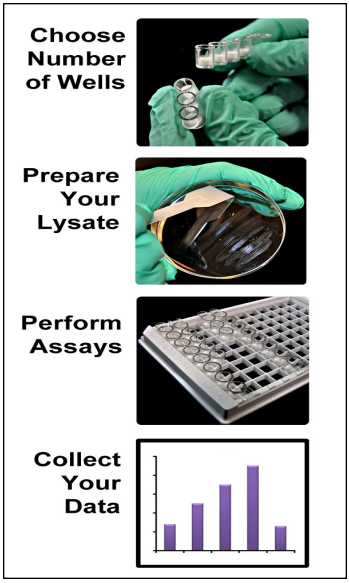
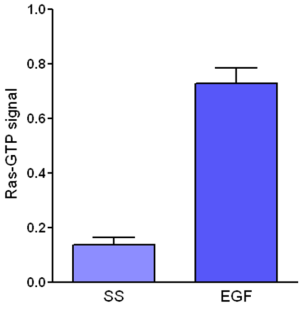


Figure 2: Typical G-LISA® Results



Ras activation by EGF measured by G-LISA®. HeLa cells were serum starved for 24 hours and treated with EGF (Cat. # CN02; 100 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.

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. (Patent# 7,763,418 B2). The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of product and components of product in research conducted by the buyer. The buyer cannot sell or otherwise transfer this product or any component thereof to a third party or otherwise use this product or its components for commercial purposes. Commercial purposes include, but are not limited to: use of the product or its components in manufacturing; use of the product or its components to provide a service; resale of the product or its components.

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

III: Kit Contents

This kit contains enough reagents for 96 assays. When properly stored, kit components are guaranteed stable for a minimum of 6 months. You can assay anywhere from 2 to 96 samples at a time for your own convenience. Table 2 summarizes the kit contents.

Table 2: Kit Contents and storage upon arrival

Reagents	Cat. # or Part # *	Quantity	Storage
96 well Ras-GTP binding plate	Part # GL64	12 strips of 8 wells	Desiccated 4°C
Anti-Ras antibody (recognizes H-Ras, K-Ras and N-Ras)	Part # GL11	1 tube, lyophilized	Desiccated 4°C
Secondary antibody - horseradish peroxidase conjugate (HRP)	Part # GL02	1 tube, lyophilized	Desiccated 4°C
Ras control protein (constitutively active H-Ras)	Part # RSCA	12 tubes, lyophilized	Desiccated 4°C
Cell Lysis Buffer	Part # GL36	1 bottle, lyophilized	Desiccated 4°C
Binding Buffer	Part # GL37	1 bottle, lyophilized	Desiccated 4°C
Wash Buffer	Part # PE38	1 tablet	Room temperature
Antigen Presenting Buffer	Part # GL45	1 bottle, 30 ml	Room temperature
Antibody Dilution Buffer	Part # 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 Stop Solution	Part # GL80	1 bottle, 8 ml	4°C
Precision Red™ Advanced Protein Assay Reagent	Part # GL50 (available as 500 ml size Cat. # ADV02)	1 bottle, 100 ml	Room temperature (4°C for long term)
Protease Inhibitor Cocktail	Cat. # PIC02	1 tube, lyophilized	Desiccated 4°C
Strip holder	Strip holder	1 plate	Room temperature

* 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.
- 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 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
96 well Ras-GTP binding plate	It is imperative to keep the plate in the zip-top bag with desiccant at all times. Reconstitution is not necessary prior to the start of the assay. The protective white powder pellet in each well of the plate may become detached from the bottom of the well during shipping. This will not affect the assay performance. Pellets should be tapped to the bottom of the well prior to resuspension.	Store desiccated at 4°C
Anti-Ras antibody	Centrifuge briefly to collect the pellet in the bottom of the tube. For each tube, dissolve the powder in 200 µl of PBS.	Store at 4°C
Secondary antibody HRP	Centrifuge briefly to collect the pellet in the bottom of the tube. Dissolve the powder in 80 µl of PBS. Do not use sodium azide in combination with this antibody as it will inactivate the HRP.	Store at 4°C
Ras control protein (12 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 100 ml of sterile distilled 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 sterile distilled water. This solution may take a few minutes to resuspend.	Store at 4°C
Wash Buffer	Reconstitute tablet in 1 L of sterile distilled 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 sterile distilled 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. Place in -70°C freezer for storage. NOTE -20°C is NOT good for storage.	Store at -70°C
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

Table 3: Component Storage and Reconstitution (Continued)

Kit Component	Reconstitution	Storage Conditions
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. Mix well and store at 4°C.	Store at 4°C
Precision Red™ Advanced Protein Assay Reagent	No reconstitution necessary.	Store at room temperature (4°C long term)
Protease Inhibitor Cocktail	Reconstitute in 1 ml of dimethyl sulfoxide (DMSO) for 100x stock.	Store at 4°C. The cocktail will freeze at 4°C

A) **Notes on Updated Manual Version– v4.0**

1) Kit replaces GL38 Wash Buffer with Bottle with PE38 Wash Buffer-tablet only. The tablet composition is identical, only the bottle was removed. This was done for space considerations only and has no effect on kit functionality.

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 Ras activation assays. Time should be taken to read this section and to carefully maintain cell lines in accordance with the guidelines given below.

For Ras activation assays, adherent fibroblast cells such as 3T3 cells should be ready at 50-70% confluency or for non-adherent cells, at approximately 6 x 10^5 cells per ml (this is a generally higher confluency than suggested for Rho family proteins that give good activation results in 3T3 cells at 30-40% confluency). Briefly, cells are seeded at 1 x 10^5 cells per ml and grown for 3-5 days. Serum starvation (see below) or other treatment should be performed when cells are approximately 50% confluent. It has been found that cells cultured for several days (3-5 days) prior to treatment are significantly more responsive than cells that have been cultured for a shorter period of time. Other cell types may require a different optimal level of confluency to show maximum responsiveness to Ras activation. Optimal confluency prior to serum starvation and induction should be determined for any given cell line (also see Appendix 5 for cell line specific references).

When possible, the untreated samples should have cellular levels of Ras activity in a "controlled state". For example, when looking for Ras activation, the "controlled state" cells could be serum starved. Serum starvation will reduce the cellular levels of active Ras and lead to a much greater response to a given Ras activator. The duration of serum starvation should be evaluated for each cell type.

Cells should also be checked for their responsiveness ("responsive state") to a known stimulus. Examples of known Ras stimuli are given in Appendix 5 and include epidermal growth factor (EGF). 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.

Typically, Ras activation is quite robust but transient in nature (see subheading D in this section). In the event that you don't see Ras activation under your experimental conditions, it is possible to evaluate the cellular response to your Ras activator by probing your cell lysates for downstream signals. See Appendix 1 for more information.

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

C) **Assay Preparation For G-LISA®**

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

Reagent	Preparation
Ras-GTP binding 96 well plate	Remove plate from 4°C and keep in its protective bag. Place on your bench at room temperature for 30 min. Do not remove the plate (or strips) from the bag until immediately prior to the experiment.
Sterile distilled 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	Determine volume of Lysis Buffer needed per culture vessel by looking at Table 5. 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). Aliquot this volume of Lysis Buffer into a clearly labeled tube and place in ice. Add 10 µl of protease inhibitor cocktail per ml of aliquoted Lysis Buffer. Mix well and leave on ice. Lysis Buffer needs to be **ice cold**. Note: you may want to supplement the Lysis Buffer with phosphatase inhibitors like sodium fluoride (25 mM final concentration) and sodium-vanadate (1 mM final concentration) if you plan to use the cell lysates to probe for downstream signals (see Appendix 1).
Ras control protein	Dissolve one tube in 500 µl Lysis Buffer and leave on ice. Use within 15 minutes. Discard any unused protein.
PBS pH 7.2	Phosphate-buffered saline is not provided in the kit. It should be prepared prior to the assay and placed on ice for at least 30 min to ensure that it is ice cold.
Anti-Ras antibody (1:50)	Have primary antibody stock ready on ice. For each 8-well strip, you will need to mix 10 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 (1:500)	Have secondary antibody stock ready on ice. For each 8-well strip, you will need to mix 1 µl antibody with 500 µl Antibody Dilution Buffer. This dilution step should be performed just prior to use as detailed in assay protocol.

Table 4: Assay Preparation for G-LISA®

Reagent	Preparation
Antibody Dilution Buffer	Place reconstituted buffer on the bench and use at room temperature.
Wash Buffer	Place on the bench and use at room temperature.
Antigen Presenting Buffer	Place on the bench and use at room temperature.
HRP Detection Reagents A and B	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.
Precision Red™ Advanced Protein Assay Reagent	Place on the bench and use at room temperature.

Preparation for G-LISA® (Continued)

D) **Timing and Intensity of Ras Activation**

Upon stimulation, Ras 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, the intensity of maximal Ras activation over “control state” (serum starved) cells is generally in the order of 2-5 fold. 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 Ras 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 at -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 time-point or condition, therefore 60-100 µl aliquots are recommended for snap freezing.

E) **Rapid processing of cells**

GTP bound (active) Ras is a labile entity and the bound GTP is susceptible to hydrolysis by Ras-GAPs during and after cell lysis, resulting in Ras inactivation. Rapid processing at 4°C is essential for accurate and reproducible results. The following guidelines are useful for rapid washing of cells.

Washing

a. Retrieve culture dish from incubator, immediately aspirate out all of the media and place firmly on ice.

b. Immediately rinse cells with an appropriate volume of ice cold PBS to remove serum proteins (see Table 5 for recommended wash volumes).

c. Aspirate off all residual PBS buffer. This is essential so that the Lysis Buffer is not diluted. Correct aspiration requires that the culture dish is placed at a steep angle on ice for 1 min to allow excess PBS to collect in the vessel for complete removal.

Cell Lysis

To avoid making too dilute or too concentrated lysate samples (<0.25 or >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. 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 that cell lysates be immediately frozen after harvest and clarification. A sample of at least 20 µl should be kept on ice for protein concentration measurement. The lysates **must** be snap frozen in liquid nitrogen and stored at -70°C. Lysates can be stored at -70°C for several months.

4. Thawing of cell lysates prior to use in the G-LISA® assay should be in a room temperature water bath, followed by rapid transfer to ice and immediate use in the assay.

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	9.6	2.0	100
60 mm dish	28	3.0	250
100 mm dish	78	10.0	700
150 mm dish	176	15.0	900
6-well cluster plate	9.5 / well	3.0	100
12-well cluster plate	4 / well	1.5	40
T-25 Flask	25	4.0	250
T-75 Flask	75	10.0	700
T-150 Flask	150	15.0	900

F) **Protein Concentration Equivalence**

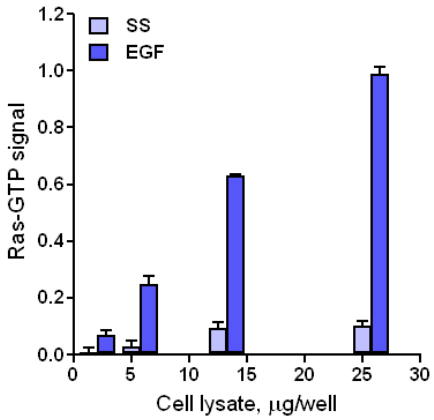
Equal protein concentration in all samples is a prerequisite for accurate comparison between samples in Ras activation assays. Cell extracts should be equalized with ice cold Lysis Buffer to give identical protein concentrations. For example, cell lysates of protein concentrations ranging from 0.3–1.3 mg/ml would all need to be diluted to 0.3 mg/ml. We highly recommend that the final concentration of equalized lysates lies between 0.25–1 mg/ml. Protein concentrations below 0.25 mg/ml often will not work with sufficient accuracy to warrant performing the assay. It is not necessary to equalize protein concentrations if the variation between them is less than 10%.

The Precision Red™ Advanced Protein Assay Reagent (Part # GL50) is included with the kit in order to measure protein concentration with a rapid one-step procedure. Precision Red™ Advanced Protein Assay Reagent is ideal for this analysis because it is 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 290 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 Appendix 4).

G) **Assay Linearity**

The assay is linear between 50 pg - 1 ng of bound activated Ras. The positive control protein is at 0.5 ng, therefore any assay readings that are more than double the positive control will be out of the linear range of the assay. In such cases you should reduce the amount of total cell protein per assay. The lower level of detection of the assay is approximately 10% above the background reading of Lysis Buffer only (0.3– 0.5). Readings lower than this require increased concentration of cell lysates to give increased protein per assay. A typical linearity test of the lysate concentration is shown in Figure 3.

Figure 3. Ras activation by EGF measured by G-LISA™.



HeLa cells were serum starved (SS) for 24 h and treated with EGF (100 ng/ml for 2 min). 25, 12.5, 5, 1.25 µg of cell lysates were subjected to the G-LISA™ assay. Absorbance was read at 490 nm. Data are background subtracted.

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) **Removal of Solutions from Wells**

Removal of solutions from the wells is accomplished by turning the plate upside down and flicking out the well contents into a waste bin. This is followed by patting the plate several times on a paper towel to get rid of residual solution. It has been found that the complete removal of solutions from the well requires a vigorous flick of the plate and a vigorous series of pats onto paper towels (5-7 hard pats). The complete removal of solution from wells between steps of the G-LISA is very important as it avoids high background readings in the buffer only wells. The buffer only wells should read between 0.3 – 0.5 at an absorbance of 490 nm. If background readings are significantly higher then a more vigorous removal of solutions from the wells may be required. The constitutively active Ras positive control wells should give a reading between 0.7 – 1.0 (background subtracted) at an absorbance of 490 nm.

J) **Plate Shaker Recommendations**

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

K) **Spectrophotometer Settings**

The majority of the work in the design of this assay has been based on the Molecular Devices SpectraMax 250. The parameters of a protocol file for the instrument are given below as a reference:

Table 6. Spectrophotometer settings. Please inquire to Technical assistance for help in setting up other machines (call 303-322-2254 for immediate help, or e-mail tservice@cytoskeleton.com for assistance within 24 h).

Parameters	Character	Contents
Wavelength	490 nm	Bandwidth 2 nm (can be ± 20 nm for filter based machines)
Protocol	End point	Standard end point assay
Shaking	Medium, orbital	5 s
Temperature	24°C	Room temperature is also fine for readings

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 2 and 3 ready to fill out as you go through the assay. Filling these out will be a good reference for you and will help facilitate any questions for technical support.

To keep the assay in the linear range we highly recommend you titrate the "controlled cell" (serum starved in most cases) lysate concentration with a G-LISA® assay. Choose the lysate concentration that gives an OD reading (after buffer blank subtraction) between 0.1-0.5. By doing this, the OD readings of "activated cell" vs. "controlled cell" are more likely to stay in the linear range.

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:

- Ras plate, at room temperature in the desiccant bag □
- Wash Buffer, resuspended at room temperature □
- Precision Red™ Advanced Protein Assay Reagent, room temperature □
- Water, 30 ml, ice cold □
- Binding Buffer, ice cold □
- HRP Stop Solution, acid added and at room temperature □
- Lysis Buffer, ice cold with protease inhibitors (see Table 5) □
- PBS, ice cold (see Table 5) □
- 1.5 ml microfuge tubes, ice cold □
- Ras Control Protein, resuspended in Lysis Buffer on ice □
- Antibody Dilution Buffer, room temperature □
- Ice buckets containing ice □

  (it is useful to have a separate ice bucket for cell harvesting)

- Cell scraper for cell collection □
- Liquid nitrogen for lysate snap freezing lysates □

STEP 2: Lysate Collection

We strongly recommend that you snap freeze your cell lysates in liquid nitrogen right after you harvest and clarify. This is especially necessary if you have many samples. It is recommended to freeze lysates in 100 µl aliquots and to save a small amount of each lysate (approximately 20 – 30 µl) for protein quantitation.

Cells Grown in Tissue Culture Vessels as Monolayers

1. Grow cells in appropriate culture conditions. It is important to keep cells in a "controlled state" prior to Ras activation. See Section V (B): Important Technical Notes.

2. Treat cells with Ras activator (or inactivator) as your experiment requires.

3. After treatment, place culture vessel on ice, aspirate media, wash with ice cold PBS. See Table 5, Section V for recommended volumes.

4. Aspirate off PBS. Tilt plates on ice for an additional 1 min and re-aspirate all remnants of PBS. Residual PBS will adversely affect the assay.

5. Lyse cells in an appropriate volume of ice-cold Cell Lysis Buffer. See Table 5, Section V for recommended volumes.

6. Harvest cell lysates with a cell scraper. It is useful to incline the culture plate for this method because the Lysis Buffer is spread thinly on the surface.

7. Transfer lysates into the pre-labeled sample tubes on ice.

8. Immediately clarify by centrifugation at 10,000 x g, 4°C for 1 min. (For well characterized experiments, the clarification step can be omitted).

9. At this point each lysate volume should not exceed 130% of the original Lysis Buffer volume.

10. Save at least 20 µl of lysate in a clean microcentrifuge tube for protein quantitation.

11. Aliquot and snap freeze the remaining cell lysates in liquid nitrogen. Store at -70°C for future use. It is recommended to aliquot into 100 µl volumes per condition (100 µl is sufficient for triplicate G-LISA assays).

12. Measure lysate protein concentrations.

    - Add 20 µl of each lysate or Lysis Buffer into disposable 1 ml cuvettes.
    - Add 1 ml of room temperature Precision Red™ Advanced Protein Assay Reagent (Part # GL50) to each cuvette.
    - Incubate for 1 min at room temperature.

- Blank spectrophotometer with the Lysis Buffer + Protein Assay Reagent cuvette at 600 nm.
- Read absorbance of lysates samples.
- Multiply the absorbance by 5 to obtain the protein concentration in mg/ml (Appendix 4).

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

14. Calculate how to equalize the cell extracts with ice cold Lysis Buffer to give identical protein concentrations. It is essential to have equal protein concentration in each sample for a successful assay. It is also important that the equalized protein concentration is not higher than 1.0 mg/ml or below 0.25 mg/ml. Specifically, we recommend the final concentration of 1 mg/ml for 3T3 and HeLa cells. It is not necessary to equalize protein concentration if the sample variation is less than 10%.

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

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

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

NOTE: If you have previously titrated the lysate and know the optimal protein concentration for your G-LISA™, you can calculate and may dilute all the samples to desired lystate concentration separately before snap freezing. Be aware of the length of time cell lysates stay on ice (should not exceed 10 min), since Ras GTP hydrolysis will occur quickly.

STEP 3: G-LISA® Assay

1. Aliquot 60 µl of Lysis Buffer into a labeled microfuge tube and dilute with 60 µl of ice cold Binding Buffer, mix well. This is your buffer blank sample. It is normal for the buffer blank to have a raw reading between 0.3 – 0.5.

2. Resuspend a fresh tube of RSCA (Ras control protein) in 500 µl of Cell Lysis Buffer (Cat# GL36). Aliquot 6 µl of RSCA into a labeled microfuge tube and dilute with 54 ul of ice cold Lysis Buffer. Dilute further by adding 60 µl of ice cold Binding Buffer. Mix well and place on ice. This is your positive control sample. NOTE: The unused Ras control protein must be discarded as it will denature. It is normal for the positive control protein to have a reading between 0.7-1.0 (after background subtraction) at an absorbance of 490 nm.

3. Take the Ras-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, seal well and place back in storage.

4. Dissolve the powder in the wells with 100 µl ice cold water, keeping the plate on ice. **NOTE:** 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.

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

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

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

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

8. Add an equal volume of ice-cold Binding Buffer to each tube. Mix well, 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 0.3 – 0.5 at an absorbance of 490 nm. If background readings are higher then a more vigorous removal of solutions from

the well should be practiced. The RSCA positive control wells should read between 0.7 – 1.0 (after subtraction of Blank) at an absorbance of 490 nm.

10. Put plate back on ice.

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

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

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

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

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

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

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

17. Place 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, patting the inverted plate 5-7 times on a stack of paper towels as outlined in step 9.

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

21. Add 50 µl of diluted anti-Ras 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 1/500 in Antibody Dilution Buffer by adding 1 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-Ras primary antibody, patting the inverted plate 5-7 times on a paper towel as outlined in step 9.

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

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

26. During the secondary antibody incubation thaw HRP detection reagents A and B in a room temperature water bath and remove as soon as they are thawed. Components A and B should be mixed in equal volumes immediately prior to use. Unused mixed solution should be discarded.

27. Immediately prior to the end of the secondary antibody incubation, mix HRP detection reagents A and B in equal volumes.

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

29. Wash the wells three times with 200 µl of Wash Buffer, patting the inverted plate 5-7 times on a paper towel as outlined in step 9.

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

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

32. Check that the wells are free of bubbles; if bubbles form, they must be removed prior to continuing.

33. 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 or visit www.cytoskeleton.com for a free Excel Template.

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

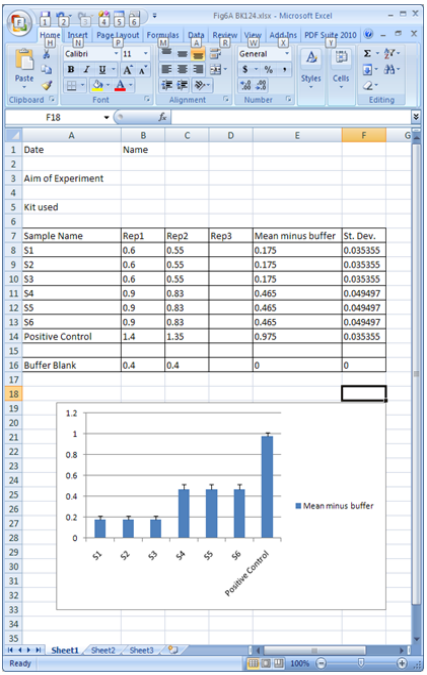
4. Enter the following formula into the first sector under “Mean”, “=average(Xn:Yn)” where X = the column designator for “rep1”, Y = column designator for “rep4”, and n= row designator of the row that you are working on. Repeat for each sector under the “Mean” header until there are sufficient rows to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

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

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

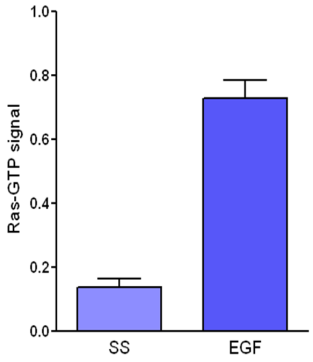
7. When the data has been entered select the Sample, Mean and Standard Deviation data sectors by the click and drag method. Then select the chart making function, in Excel this looks like a clickable square with a mini-bar chart inside. This will guide you through the chart making process with the data you have selected. Choose “column chart” initially, designate the Mean numbers for input values. The Standard Deviation column for the y-axis error bars needs to be designated after the Mean numbers chart is made. This is achieved by double clicking on the graph bars, and selecting the “Y-axis error” tab, then entering the location of the Standard Deviation data by clicking the “Custom” option and selecting the area in the worksheet. E-mail tservice@cytoskeleton.com for a free Excel Template. An example of a typical Excel layout and data plot is shown in Figures 4 and 5.

Figure 4: Typical Excel Layout



	A	B	C	D	E	F
1	Date	Name				
2						
3	Aim of Experiment					
4						
5	Kit used					
6						
7	Sample Name	Rep1	Rep2	Rep3	Mean minus buffer	St. Dev.
8	S1	0.6	0.55		0.175	0.035355
9	S2	0.6	0.55		0.175	0.035355
10	S3	0.6	0.55		0.175	0.035355
11	S4	0.9	0.83		0.465	0.049497
12	S5	0.9	0.83		0.465	0.049497
13	S6	0.9	0.83		0.465	0.049497
14	Positive Control	1.4	1.35		0.975	0.035355
15						
16	Buffer Blank	0.4	0.4		0	0

Figure 5: Typical G-LISA® Results



Ras activation by EGF measured by *G-LISA*™. HeLa cells were serum starved for 24 hours and treated with EGF (100 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.

Note: Refer to our website for timely updates on technical tips for G-LISA™ assays.

IX: Troubleshooting

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. Well contents will appear sticky and opaque. 4. A step or component of the assay was omitted. 5. The HRP reaction was not developed for long enough.	1. Process samples quickly on ice. Snap freeze sample aliquots. 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 cover on the plate. 4. Read instructions carefully. 5. The HRP reaction should be allowed to develop for 15 min at room temperature. HRP Stop Solution should be added prior to reading at 490 nm.
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. 2. Follow the instructions for the washing in the manual.
Background readings are high (>0.50)	Inefficient removal of solutions from G-LISA wells.	Background should read between 0.30 – 0.50. Complete removal of solutions from the wells is required to produce a clean assay. Vigorous flicking and patting of the inverted plate is required to completely remove solutions from the wells after each step is complete. See Important Technical Notes and the G-LISA method for details.
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-Ras level is too high. 5. Tissue culture cells not correctly serum starved. 6. Temperature of lysis and incubation is not 4°C. 7. The Binding buffer is not pre-cooled at 4°C.	1. Purchase a fresh vial of inducer. 2. Read instructions carefully and compare with your Experiment Record Sheet 3. The linear range of the assay is 50 pg – 1 ng Ras. 4. Titrate down the amount of extract to be added. 5. Details of how to serum starve cells is given in Appendix 5 references. 6. Lyse cells on ice and keep all cell lysis and distilled waters on ice. 7. Make sure the buffer was stored in the fridge and kept on ice before use.
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	Positive control protein was stored after reconstitution.	1. Use a fresh tube of Ras positive control protein each time. There are 12 per kit.
Serum starved control sample is reading at or slightly below buffer only background	In many cases, serum starvation will result in negligible levels of active, GTP-bound, Ras and the signal will be close to or even slightly below background. This makes it difficult to get a fold increase for background subtracted samples.	1. If this is a problem you should use lysates of a higher concentration to get the serum starved signal above background.

Section X: References

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2. Bourne, H.R., Sanders, D.A. & McCormick, F. 1990. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature*. **348**, 125-132.
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4. Schlichting, I. et al. 1990. Time-resolved conformational change in Ha-Ras p21 protein on GTP hydrolysis. *Nature.* **345**, 309-315.
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6. De Rooij, J. & Bos, J.L. 1997. Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. *Oncogene*. **14**, 623-625.
7. Herrmann, C., Martin, G.A. & Wittinghofer, J. 1995. Quantitative analysis of the complex between p21ras and the Ras-binding domain of the human Raf-1 protein kinase. *J. Biol. Chem.* **270**, 2901-2905.
8. Taylor, S.J., Resnick R.J. & Shalloway, D. 2001. Nonradioactive determination of Ras-GTP levels using activated ras interaction assay. *Method Enzymol*. **333**, 333-342.

Appendix 1: Evaluating the "controlled" and "responsive" state of the cells

If the G-LISA results suggest that there was no apparent activation of Ras under your assay conditions, it may be worth conducting another experiment to probe the cell lysates for signals downstream of Ras. There are generally two scenarios where this could happen. It should be possible to resolve this issue in both scenarios by probing for downstream signals in the cell lysates while optimizing the conditions of cell growth or treatment. It is important, however, that your cell lysates were prepared using Lysis Buffer that contains both protease inhibitors and phosphatase inhibitors such as NaF (25 mM) and sodium vanadate (1 mM).

Scenario 1: Both the "controlled" state and "responsive" state cell lysates are giving high absorbance readings relative to the background wells and they are not significantly different from each other.

- Typically this means you need to optimize the conditions for your controlled state, which may mean optimizing your serum starvation conditions depending on your experimental design

- Consider running a Western blot to probe for signals downstream of Ras while varying your serum starvation conditions (or other conditions if relevant). When you have found growth conditions that minimize the basal level of Ras pathway activation as determined by the downstream signal, repeat your original experiment.

Scenario 2: Both the "controlled" state and "responsive" state cell lysates are giving roughly equal absorbance readings to the background wells.

- Results of this nature can occur for several reasons, some of which are listed in the Troubleshooting section. Other reasons include…
  1. You've missed the optimal window for Ras activation by your chosen stimulus. Ras activation can be very transient and you may have missed the peak activation timepoint when preparing your cell lysates.
  2. Your Ras activator is not biologically active. The "controlled" state of the cells is excellent, but your cells are not responding to the non-functional Ras activator
  3. Your Ras activator does not work in the cell type you've chosen.

- Consider running a Western blot to probe for signals downstream of Ras while varying your activation conditions. When you have found growth conditions that maximize Ras pathway activation as determined by the downstream signal, repeat your original experiment.

See the next page for antibody recommendations to probe your cell lysates for Ras downstream signaling

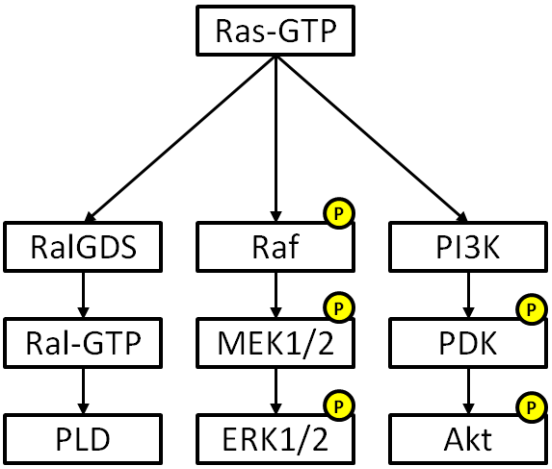


Figure 1. A simplified representation of Ras-GTP signaling in cells. The yellow circles with the letter P inside reflect downstream phosphorylation events that are possible to monitor by Western blot.

Downstream signals to consider: Figure 1 is a highly simplified representation of the 3 main Ras-GTP signaling pathways. High quality antibodies are commercially available for key phosphorylation events that occur downstream of Ras in the Raf / MEK / ERK and PI3K / PDK / Akt pathways, which provide a means of probing your cell lysates for downstream Ras pathway activation by Western blot. The specific phosphorylation events are listed below to help guide your purchase of an appropriate antibody.

- **Phospho-ERK1/2**: The appropriate antibody will recognize ERK1/2 when phosphorylated on Thr202 / Tyr204. These phosphorylation sites are among the most commonly used markers for downstream Ras signaling.
- **Phospho-MEK1/2**: The appropriate antibody will recognize MEK1/2 when phosphorylated on Ser217 / Ser221.
- **Phospho-Raf**: The appropriate antibody will recognize Raf when phosphorylated on one of the several sites that are phosphorylated during Raf activation (e.g. c-Raf: Ser338, Tyr341, Thr491, Ser494, Ser497 and/or Ser499).
- **Phospho-Akt**: The appropriate antibody will recognize Akt when phosphorylated on Thr308.
- **Phospho-PDK**: The appropriate antibody will recognize PDK when phosphorylated on Ser241.

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. How long were the ice cold solutions on ice before lysis?	…….. Min
4. Time that cultures were removed from incubator?	……. am or pm
5. Was Binding Buffer added?	…….. Y or N
6. Time that binding reactions were placed on the shaker?	…….. am or pm
7. Did you add 50 µl of extract per well?	…….. Y or N
8. What locations are the 50 µl Lysis Buffer controls?	……. Wells
9. What speed and time was the shaking for the binding reaction?	…… rpm ……min
10. How long did you wait after the post-binding wash step?	……. s or min
11. What was the time when the anti-Rho 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

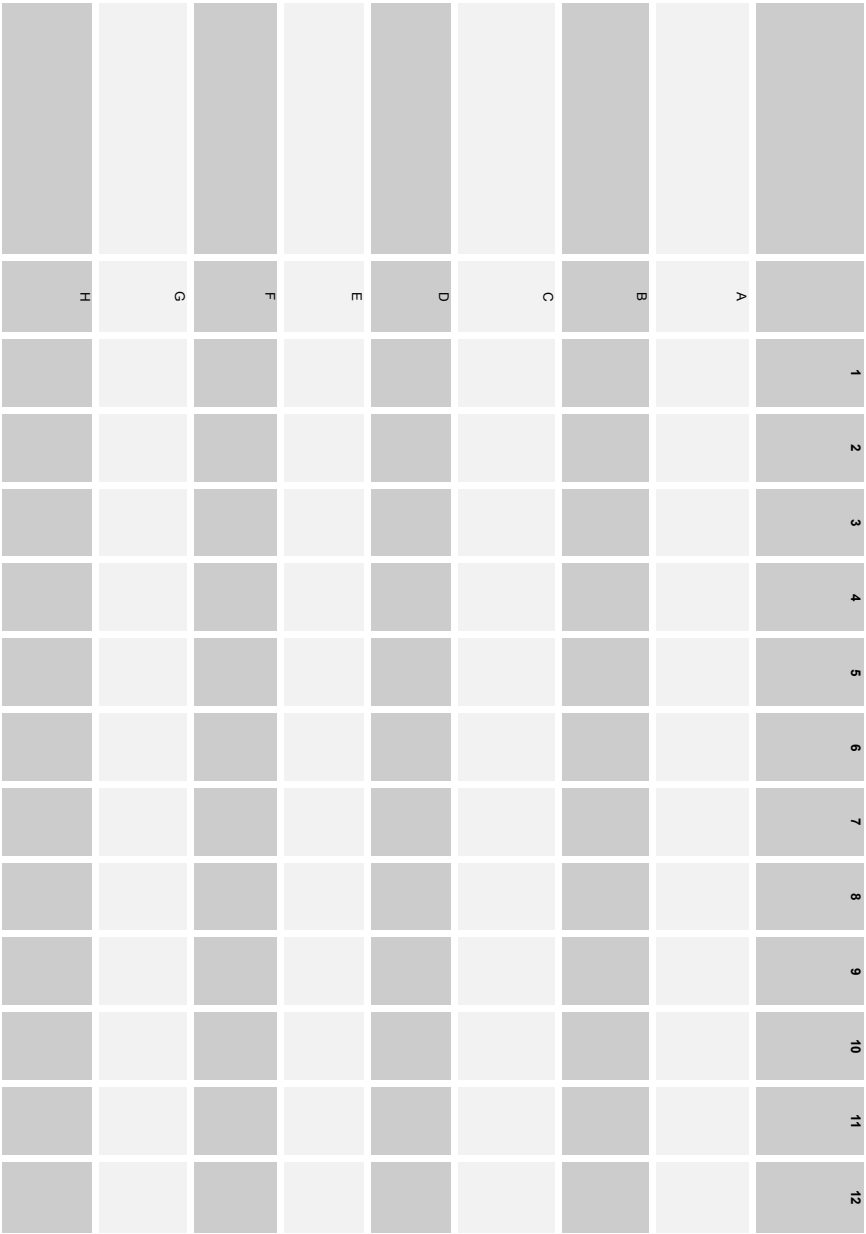
Technical Assistance: call either 303-322-2254 or e-mail tservice@cytoskeleton.com.

Appendix 3: Plate Record Template

Name of experiment: ……..

Date of experiment: ……

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



Appendix 4: Protein Quantitation (with Precision Red Reagent)

Background

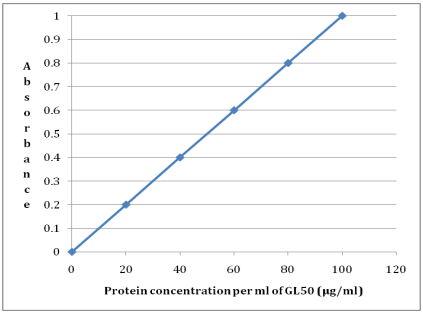
The Precision Red Advanced Protein Assay Reagent is a simple one step procedure that results in a red to purple/blue color change characterized by an increase in absorbance at 600 nm. The reagent is supplied in this kit as Part # GL50. It is also sold separately as Cat. # ADV02.

The assay exhibits low variance in readings between different proteins of the same concentration and high reproducibility of the colorimetric response. This allows one to utilize a generally applicable standard curve (Fig. 1) for protein quantitation. The assay can also be performed in approximately 1-2 minutes. These properties are particularly valuable when applied to the labile lysates required for G-LISA assays.

Quick Protein Concentration Method for 1 ml Cuvette (recommended)

- Add 20 µl of each lysate or Lysis Buffer into disposable 1 ml cuvettes.
- Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Part # GL50) to each cuvette.
- Incubate for 1 min at room temperature.
- Blank spectrophotometer with 1 ml of GL50 plus 20 µl of Lysis Buffer at 600 nm.
- Read absorbance of lysate samples.
- Multiply the absorbance by 5 to obtain the protein concentration in mg/ml

Fig. 1: Standard Curve for Protein Quantitation in a 1ml Cuvette



Legend: The standard curve shown in Fig. 1 represents the average absorbance reading of several common proteins (e.g., actin, BSA, casein) measured in a 1 ml cuvette format using 1 ml of GL50 reagent. The protein reading pathlength for a cuvette is 1 cm. Linear range of this assay is 0.05 - 0.6.

Example Calculation

Assume a 20 µl sample of cell lysate added to 1 ml of GL50 gives an absorbance reading of 0.1.

$$C = \frac{A}{\varepsilon \times l} = \frac{0.1}{10 \times 1} \times 50 = 0.5 \text{ mg/ml}$$

Where c = protein concentration (mg/ml), A = absorbance reading, l = pathlength (cm), ε = extinction coefficient ($[mg/ml]^{-1}$ cm^{-1}) and the multiplier of 50 is the dilution factor for the lysate in GL50 (20 µl lysate in 1 ml GL50).

Thus, for a 20 µl sample in 1 ml GL50, the equation becomes $C = A \times 5$

For a 10 µl sample in 1 ml GL50, the equation becomes $C = A \times 10$

Quick Protein Concentration Method for 96 Well Plate

- Add 10 µl of each lysate or Lysis Buffer into the well of a 96 well plate.
- Add 290 µl of Precision Red™ Advanced Protein Assay Reagent (Part # GL50) to each well.
- Incubate for 1 min at room temperature.
- Blank spectrophotometer with 290 µl of GL50 plus 10 µl of Lysis Buffer at 600 nm.
- Read absorbance of lysate samples.
- Multiply the absorbance by 3.75 to obtain the protein concentration in mg/ml

96 Well Plate Method

The linear range of this assay is 0.05 - 0.4 and is recommended when lysates are below the linear range of the 1 ml cuvette method. The pathlength for 96 well plate readings is 0.8 cm, hence the equation is modified as shown in the example below:

Example Calculation for 96 Well Plate Measurement

Assume a 10 µl sample of cell lysate added to 290 µl of GL50 gives an absorbance reading of 0.1

$$C = \frac{A}{\varepsilon \times l} = \frac{0.1}{10 \times 0.8} \times 30 = 0.375 \text{ mg/ml}$$

Where c = protein concentration (mg/ml), A = absorbance reading, l = pathlength (cm), ε = extinction coefficient ($[mg/ml]^{-1}$ cm^{-1}) and the multiplier of 30 is the dilution factor for the lysate in GL50 (10 µl lysate in 290 µl GL50).

Thus, for a 10 µl sample in 290 µl GL50, the equation becomes C = A x 3.75

For a 5 µl sample in 295 µl GL50, the equation becomes C = A x 7.5

NOTE: The protein concentrations generated by using the standardized protein curve (Fig.1) will generate approximate lysate concentrations. Data will be highly reproducible from lysate to lysate and will generate excellent values for relative concentrations of a series of lysates. It should be noted for G-LISA® assays, and activation assays in general, the relative protein concentration between experimental extracts is far more important than the absolute protein quantitation. However, if desired, one can create a standard curve using BSA or IgG protein standards for each experiment. The standard curve should be performed prior to lysate preparations due to the labile nature of the lysates.

Appendix 5: Known Ras Activators

Activator	Treatment	Cell Line Used	Response	Type of Assay	Ref.
Epidermal Growth Factor	100 ng/ml 5 minutes	HeLa	Dose dependent activation	Raf1-RBD pulldown assay	1
Hepatocyte Growth Factor	100 ng/ml 5 minutes	HeLa	Dose dependent activation	Raf1-RBD pulldown assay	1
IL-3	50 ng/ml 5 minutes	BaF3	14 fold activation after 5 minutes	Raf1-RBD pulldown assay	2
αCD3 + αCD28	5 μg/ml each 10 minutes	Jurkat	Dose dependent activation	Raf1-RBD pulldown assay	3
PMA ionomycin	100 ng/ml 500 ng/ml 10 minutes	Jurkat	Dose dependent activation	Raf1-RBD pulldown assay	3

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2 Satoh T. et al. 1993. Platelet-derived growth factor receptor mediates activation of ras through different signaling pathways in different cell types. *Mol. Cell. Biol*. **13**, 3706-3713.

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